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# Development of new screening techniques to assess bioavailability of soy isoflavones: use of a hamster model and genomic techniques to identify human fecal microorganisms associated with isoflavone disappearance

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**Development of new screening techniques to assess bioavailability of soy isoflavones:  
use of a hamster model and genomic techniques to identify human fecal  
microorganisms associated with isoflavone disappearance**

by

Mathieu Renouf

A dissertation submitted to the graduate faculty  
in partial fulfillment of the requirements for the degree of

**DOCTOR OF PHILOSOPHY**

Major: Nutrition

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## ABSTRACT

Isoflavones are found in the human diet almost exclusively in soybeans and soy products. Because of their structural similarities to the main female hormone estrogen, they have been extensively studied for improving health and preventing several chronic diseases. Isoflavones have been studied for their protecting effects against cancer, osteoporosis, cardiovascular disease, hypercholesterolemia and menopause-related symptoms. Little is known about health-adverse and toxic effects associated with these compounds. Moreover, the need to better understand bioavailability of isoflavones is essential with respect to their overall metabolism and modes of actions. Two aspects of isoflavone bioavailability are investigated in the present research project: establishment of a human-relevant animal model to study bioavailability and subsequent health effects as well as identifying the microorganisms influencing the extensive and variable degradation of isoflavone in the human large intestine to obtain a better screening tool of individual capability to degrade these compounds, which may greatly affect health outcomes measured in clinical trials.

Golden Syrian hamsters were investigated in three studies to attempt the validation of this model as relevant to human for isoflavones bioavailability and their cholesterol lowering effect. Apparent absorption of isoflavones, as reflected in urinary excretion, showed great sex differences in daidzein, glycitein and genistein; females were 2-3 fold higher excreters than males. Fecal excretion did not exceed 0.5% ingested dose and did not correlate with urinary excretion. Cecal but not fecal *in vitro* isoflavone degradation rates were strongly correlated with individual urinary excretion levels of daidzein and genistein. However, glycitein urinary excretion and the lack of relationship to urinary excretion showed an unstable pattern of bioavailability compared to daidzein or genistein. Finally, hamsters excreting large amounts of isoflavone in their urine had significantly lower levels of total cholesterol compare to animals excreting smaller amounts of daidzein, genistein and total isoflavone. Thus, we concluded that sex-difference, similar pattern of urinary excretion between females and women, differences between sex (females>males) and isoflavone (daidzein>genistein) and clustering of animals as high excreters/low degraders and low excreters/high degraders were common features shared with humans that may lead to validating hamsters as a good model to study bioavailability of isoflavones. Lower levels of excretion in male hamsters compared



to human males, instability in glycitein metabolism and concordance between human fecal and hamster cecal but not hamster fecal bacterial activity were observed differences that may contraindicate the use of this animal model.

To understand the complexity of human fecal microflora related to isoflavone disappearance (apparent degradation), we coupled molecular biology with microbiology techniques. The use of polymerase chain reaction coupled with denaturing gradient gel electrophoresis allowed the profiling both qualitatively and quantitatively of fecal microbial profiles based on each strain's 16S DNA. When comparing gut microbial profiles to in vitro fecal degradation of isoflavones, 7 bands of interest were identified when focusing on intra-individual variability of fecal glycitein degradation over a one month interval. Screening of 33 subjects (13 females and 20 males) to determine high versus low fecal isoflavone degraders (determined by ranking and clustering of individual degradation rates for each isoflavone) gave 5 bands of greater intensity in the 4 highest degraders compare to the 4 lowest glycitein degraders, 5 bands for genistein and no bands for daidzein.

Sequencing and identification of microorganisms using Basic Local Alignment Search Tool (BLAST) gave matches to *Bacteroides*, *Prevotella*, *Bacteroidales* and *Clostridiales* species (*Bacteroides fragilis*, *Bacteroides uniformis*, *Bacteroides ovatus*, *Bacteroides vulgatus*, *Bacteroides thetaiotaomicron*, *Bacteroides eggerthii*, *Porphyromonas gingivalis*, *Prevotella pallens*, *Prevotella ruminicola*, *Prevotella oralis*, *Prevotella veroralis*, *Ruminococcus obeum*, *Fusobacterium prausnitzii*, *Bacteroides acidifaciens* and *Tannerella Forsythensis*). *Eubacterium ramulus* and *Clostridium orbiscindens* were also selected from previously published literature. Investigations of these strains were done using a fecal suspension matrix in which nutrient availability was either rich (attempting to mimic a rapid gut transit time and expected low degradation rates) or poor (to mimic long gut transit time; high degradation rates). Nutrient rich media contained several nutrient sources including sterilized rumen fluid, peptone, brain heart infusion powder, yeast extract and sucrose whereas nutrient poor media was obtained by incubating 1g of feces overnight in brain heart infusion media. Species increasing significantly isoflavones degradation in both nutrients were *B. acidifaciens* ( $143.6 \pm 16$  vs.  $180.3 \pm 5.0$  band intensity arbitrary units in low vs. high degraders;  $p < 0.05$ ), *T. forsythensis* ( $120.5 \pm 18.1$  vs.  $187.5 \pm 22.5$  intensity arbitrary units;

$p < 0.05$ ), *E. ramulus* and *C. orbiscindens* (both selected from previous literature), and to some extent *P. pallens* ( $123.0 \pm 2.8$  vs.  $170.0 \pm 1.4$  intensity arbitrary units;  $p < 0.05$ ) and *B. uniformis* ( $171.3 \pm 21.0$  vs.  $210.0 \pm 10.8$  intensity arbitrary units;  $p < 0.05$ ) that will deserve further analysis. These species were of greatest interest and therefore hypothesized to be present in both high and low excretors with greater proportions of these strains in high compared to low degraders, as reflected by DGGE analysis. These findings support the idea that low and high degradation patterns may share common microorganisms, some being more active in one pattern of degradation compared to the other. In addition, other species investigated showed significant effect in increasing isoflavones degradation nutrient-poor media only (*P. pallens*, *P. oralis*, *B. eggerthii* and to some extent *B. vulgatus*, *B. fragilis*, *P. ruminicola*, *P. veroralis* and *F. prausnitzii*), suggesting that a wider range of species may be capable of increasing isoflavone when nutrients are lacking, also explaining the phenomenon of high degradation in humans.

## GENERAL INTRODUCTION

### Introduction

Isoflavones are bioactive components found mostly in soybeans and soy products. Amounts of isoflavones found in food vary from 1 to 3 mg/g food and are found in two major forms. The glucoside forms are daidzin, genistin and glycitin, mostly found in non-processed foods while the aglycone forms are daidzein, genistein and glycitein, mostly found in processed and fermented soy foods. Small amounts of acetyl and malonyl isoflavones also occur in soyfoods. Special focus on isoflavones has been brought over the years because of their potential in improving health and preventing chronic diseases. Their chemical structures are close to estrogen, the main female hormone, thus classifying isoflavones as phytoestrogens. The range of health effects is wide and includes prevention of cancer, osteoporosis, hypercholesterolemia and menopausal symptoms as well as potential beneficial effects on immune function and menstrual cycle. Toxic and adverse effects are controversial, with little substantial evidence of any major concern so far for individuals who ingest large amounts of isoflavone on a daily basis. To study these health effects, animal models (from rats to monkeys) have been extensively used.

Isoflavone bioavailability is quite complicated and defined by an intricate scheme of absorption, plasma and urinary kinetics and gut microbial metabolism and degradation. We now know that isoflavone glucosides undergo cleavage of their sugar moiety by intestinal  $\beta$ -glucosidases to be absorbed mostly in the stomach and the small intestine, as evidenced by a rapid peak appearance in plasma (less than 6 h after ingestion). After absorption, isoflavones undergo intestinal and hepatic biotransformation mainly to glucuronide conjugates. Clearance of isoflavone occurs within 24-48 h after ingestion, but the amounts excreted in urine and feces account for less than half of the amount ingested. Most of the conjugates are excreted through bile back into the intestine. Deconjugation, mostly by bacteria in the large intestine, is necessary for reabsorption. Bacteria are also responsible for degrading and producing by-products and metabolites of isoflavones. Colonic microbial metabolism has been established as a critical factor in understanding the concept of isoflavone

bioavailability. This extent of microbial degradation is wide and inversely correlated to urinary and fecal excretion in humans. Establishment of phenotypes of isoflavone bioavailability was introduced less than 10 years ago to categorize individuals as high, moderate or low degraders. However, despite a greater understanding of gut microbial degradation and metabolism/bioavailability of isoflavones, the actual bacterial species responsible for this degradation remain unknown.

With regard to health-related effects of isoflavones investigated in animals, relevance of the data extrapolated to humans underlies the assumption that bioavailability is similar between the animal model studied and humans. To our knowledge, there is very limited evidence about the validity of a specific model in terms of bioavailability or metabolism that could be suitable for data extrapolation to humans.

With respect to what is or is not known about bioavailability of isoflavones, we decided to investigate in greater detail two of the main questions still limiting the overall knowledge of isoflavone mode of action and metabolism. First, it is essential to validate an animal model with close resemblance to humans with respect to bioavailability so that health-related effects investigated in that particular model would be of greater relevance. We chose Golden Syrian hamsters, because of a similar cholesterol metabolism compared to humans. If hamsters do resemble humans in metabolizing isoflavones, then health-related effects studied in this model would be of greater relevance. Second, identifying gut microorganisms responsible for isoflavone degradation would fill one of, if not the greatest gaps in understanding bioavailability. It would provide a useful tool to screen people for their bacterial isoflavone degradation, which would predict the overall pattern of apparent absorption and bioavailability. This would be the first step in a long process of limiting these species in the gut to improve absorption and possibly promote health effects. Limiting isoflavone-degrading microorganisms would also limit inter-individual variation in bioavailability so that a dose given orally would be closer to the dose actually exerting a physiological effect.

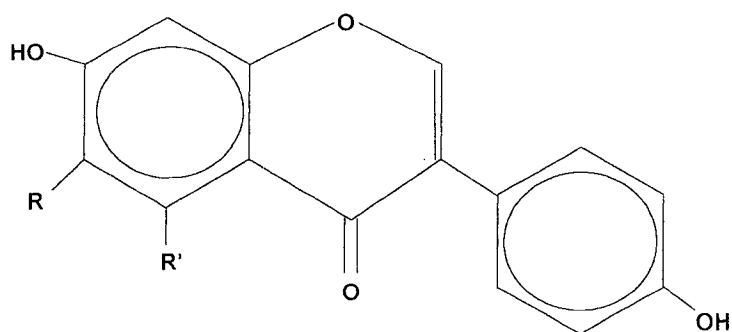
**Dissertation organization**

This dissertation contains a general introduction, a literature review focusing mainly on health-effects, bioavailability and metabolism of isoflavones, and composition and metabolism of the human gut microflora. All three papers “Isoflavone excretion phenotypes influence plasma cholesterol in Golden Syrian hamsters” and “Bioavailability of soy isoflavones in Golden Syrian hamsters” and “Identification of isoflavone-degrading microorganisms in humans using polymerase chain reaction and denaturing gradient gel electrophoresis” will be submitted to the *Journal of Nutrition*.

## LITERATURE REVIEW

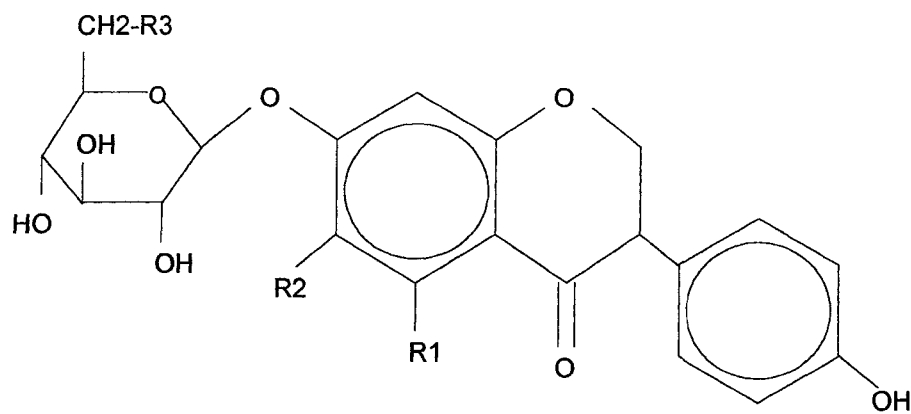
### A. Structures, sources and dietary intake of isoflavones

Isoflavones are found almost exclusively in soybeans and soy products (Wang and Murphy. 1994; Wang and Murphy. 1996). Soybean isoflavones are daidzin, genistin and glycitin, the glucoside form of daidzein, genistein and glycitein, respectively (Figures 1 and 2). Other isoflavone forms, such as malonyl- and acetyl-isoflavones also occur in soy foods (Wang and Murphy. 1994; Wang and Murphy. 1996).



Compounds	R	R'
daidzein	H	H
genistein	H	OH
glycitein	OCH <sub>3</sub>	H

Figure 1: Isoflavone chemical structure



Compound	R1	R2	R3
daidzin	H	H	-OH
6"-O-acetyldaidzin	H	H	-OCOCH <sub>3</sub>
6"-O-malonyldaidzin	H	H	-OCOCH <sub>2</sub> COOH
genistin	OH	H	-OH
6"-O-acetylgenistin	OH	H	-OCOCH <sub>3</sub>
6"-O-malonylgenistin	OH	H	-OCOCH <sub>2</sub> COOH
glycitin	H	OCH <sub>3</sub>	-OH
6"-O-acetylglycitin	H	OCH <sub>3</sub>	-OCOCH <sub>3</sub>
6"-O-malonylglycitin	H	OCH <sub>3</sub>	-OCOCH <sub>2</sub> COOH

Figure 2: Chemical structure of the different isoflavone forms found in soy foods

According to the US Department of Agriculture/Iowa State University Isoflavone Database (1999) and analytical studies conducted by Murphy et al. (1999), isoflavones are found almost exclusively in soybeans and soy products (Table 1). Exceptions include alfalfa and clover sprouts. Isoflavone concentrations in soybean foods are close to 150 mg/100g (Table 1).

Food item	Isoflavone	mean	SD	n
Miso	daidzein	16.13	4.36	7
	genistein	24.56	4.23	9
	glycitein	2.87	0.47	3
	total isoflavone	42.55	9.18	7
Instant beverage, soy powder not reconstituted	daidzein	40.07	6.19	6
	genistein	62.18	2.78	6
	glycitein	10.9	0.14	4
	total isoflavone	109.51	4.11	6
Soy flour, full fat, row	daidzein	71.19	6.95	20
	genistein	96.83	7.38	21
	glycitein	16.18	2.65	7
	total isoflavone	177.89	12.57	21
Soymilk, fluid	daidzein	4.45	0.75	14
	genistein	6.06	0.84	16
	glycitein	0.56	0.09	5
	total isoflavone	9.65	1.76	14
Soy protein isolate	daidzein	33.59	5.99	14
	genistein	59.62	6.68	14
	glycitein	9.47	1.81	11
	total isoflavone	97.43	11.11	14
Soybeans, mature seeds, dry roasted	daidzein	52.04	14.04	7
	genistein	65.88	14.89	8
	glycitein	13.36	5.94	5
	total isoflavone	128.35	33.38	7
Tempeh	daidzein	17.59	3.13	6
	genistein	24.85	5.47	6
	glycitein	2.1	0.67	3
	total isoflavone	43.52	8.34	6
Tofu, firm, prepared with calcium sulfate and nigari	daidzein	9.44	1.68	6
	genistein	13.35	2	7
	glycitein	2.08	0.15	4
	total isoflavone	24.74	3.77	6

Table 1: Mean individual and total isoflavone levels in selected soy foods (mg/100 g edible portion), standard deviation (SD) and total number of individual values (n) (Adapted from US Department of Agriculture/Iowa State University database on the isoflavone content of foods. 1999).



The differences observed in isoflavone content from one soyfood to another are partly due to food processing losses of isoflavones. Wang and Murphy (1996) analyzed the isoflavone content in the different steps of making tempeh, soymilk and tofu from whole soybeans. Using HPLC as an analytical method, they observed a 76% decrease in isoflavone content during production of tempeh, mostly due to the soaking and cooking step. They also observed that fermentation increased the aglycone content. Significant losses of isoflavones also occurred during coagulation (44%) in tofu processing as well as alkaline extraction (53%) during soy protein isolate production. On the other hand, the isoflavone content after processing of soymilk was not significantly different from raw soymilk. Although soy foods are the main contributor of isoflavones in the diet, isoflavone content varied from one variety of soybean to another or within the same variety from one year to another (Wang and Murphy, 1994). Therefore, accurately analyzing isoflavone intake is a challenge.

Due to the low consumption of soy products in the Western diet, isoflavone intake has been estimated not to exceed 1-2 mg/d per person in the US (Munro et al. 2003). On the other hand, a traditional Asian diet, usually associated with consumption of soybeans or soy foods, may provide about 50 mg/d of isoflavones (Nagata et al. 1998). Overall, isoflavone consumption in a Western diet has been evaluated to be ~0.01 mg/kg body weight/d whereas in an Asian diet, isoflavone consumption may reach up to 0.5 mg or ~1.8  $\mu$ mol/kg body weight/d (Munro et al. 2003).

## **B. Isoflavones and health**

Protection from certain chronic diseases (cancer, osteoporosis) and health-related factors (blood lipid profile, consequences of menopause) has been extensively studied with respect to isoflavones, either alone or in combination with a food matrix. The discrepancies in protocols and methods along with a wide range of isoflavone doses applied across studies lead to difficult comparisons of data and conclusions. Whenever possible and available, doses applied to either humans or animals will be converted to mole concentrations of individual or dose of total isoflavone/kg body weight/d in order to make comparison across studies more relevant. Discussion of these intakes with respect to an established “normal”

dietary intake of isoflavones from soyfoods in humans will also be included. An Asian diet provides up to 0.5 mg total isoflavone/kg body weight/day, which corresponds to  $\sim 1.8 \mu\text{mol}$  total isoflavone/kg body weight (BW)/day (d). The following is a review of isoflavones with respect to cancer, blood lipid profile, immune function, osteoporosis, menstrual cycle, menopause, infants and toxicity in humans and/or animals and/or in vitro.

## 1. Isoflavones and cancer

According to the American Cancer Society (2000), cancer is the second leading cause of death in the US (25% of all deaths). In women, the most prevalent types of cancer are lung, breast, colon, ovary, pancreas, stomach and liver whereas in men, the main cancer sites are lung, prostate, colon, pancreas, stomach and liver. Nutrition has been extensively studied with respect to cancer because dietary compounds may protect against this chronic disease. Soy foods and the numerous bioactive compounds they contain are now widely studied for their possible cancer-preventive effect.

### a. Human studies

In humans, the role of soy in preventing cancer has emerged from epidemiological studies. Asians consuming a traditional Asian diet (high in soy products) have lower cancer rates compared to Caucasians consuming a typical western diet.

In a case control study, Zheng et al. (1999) looked at urinary phenol and total isoflavonoid as markers of soyfood intake in women with or without breast cancer from Shanghai (n=60/group; 25 to 64 years of age with aged-matched control subjects) and found a significant difference in breast cancer incidence between the highest (n=35;  $>18.66 \text{ nmol}$  isoflavone/mg creatine) and lowest (n=14;  $<5.58 \text{ nmol/mg creatine}$ ) tertile of urinary phenol and isoflavonoid excretion ( $p=0.04$ ).

Dai et al. (2003) studied urinary isoflavones excretion and breast cancer risk with respect to hormonal levels, body mass index (BMI) and waist:hip ratio (WHR) (n=117 case-control pairs of postmenopausal women from Shanghai). Mean age of subjects was  $\sim 56$  years

of age. They found a negative relationship between urinary excretion of isoflavone and breast cancer risk for subjects with higher BMI ( $>25$ ;  $p$  for trend  $=0.06$ ), higher WHR ( $>0.84$ ;  $p=0.02$ ), higher plasma estradiol ( $>5.73$  pg/mL;  $p=0.01$ ), lower blood estrone sulfate ( $<0.96$  ng/mL;  $p=0.01$ ) and lower sex hormone binding globulin concentrations ( $<81.4$  nmol/L;  $p=0.03$ ). Therefore isoflavone intake was considered a key factor in decreasing risks of breast cancer, but other parameters must be considered in the analysis (blood hormonal levels, BMI, WHR) in order to make the correlation statistically significant.

With respect to endometrial cancer risks, Horn-Ross et al. (2003) performed a case-control study of 500 women who developed endometrial cancer and 470 healthy women. They found a negative relationship between cancer risk and isoflavone intake estimated from a food frequency questionnaire. Isoflavone intake was divided in quartiles and comparison between the highest ( $>2726$  ug total isoflavone/d or  $0.14$   $\mu\text{mol/kg BW/d}$ ) and the lowest quartile ( $<1150$  ug/d or  $0.06$   $\mu\text{mol/kg BW/d}$ ) showed the strongest correlation between cancer protection and isoflavone consumption (adjusted odds ratio $=0.59$ ; 95% confidence interval $=0.37$  to  $0.93$ ;  $p$  value for trend $=0.2$ ). BMI was not a significant factor in this study ( $p$  value for interaction $=0.27$ ), which differs from Dai et al. (2003). However, levels of isoflavones were assessed as intake and not urinary excretion, thereby bypassing bioavailability that could have greatly influenced the measured outcomes.

Prostate cancer was also studied epidemiologically with respect to isoflavone intake by Lee et al. (2003). Chinese subjects with ( $n=133$ ) or without ( $n=265$ ) prostate cancer were assessed for their soy food and isoflavone intake using a food frequency questionnaire. A significant negative correlation ( $p$  value for linear trend $=0.032$ ) was observed between prostate cancer risk and intake of tofu.

Correlations between equol, the main daidzein metabolite, and cancer have also been investigated in epidemiological studies. Akaza et al. (2004) examined the prevalence of prostate cancer and equol production in Japanese (133 patients, 162 controls), Koreans (61 patients, 61 controls) and Americans (24 patients, 21 controls). The percentage of equol producers was significantly higher in controls vs. patients in Japanese (46 vs. 29%;  $p=0.004$ ) and Koreans (59 vs. 30%;  $p=0.001$ ). In Americans, equol producers were similar between patients (17%) and controls (14%), but plasma daidzein and genistein measured in this

population was ~10 times lower than in Japanese or Korean groups. The authors concluded that a diet high in soy products coupled with the ability to produce equol may be more significant in protecting against prostate cancer.

Despite significant data negatively correlating soy food intake and the prevalence of cancer, isoflavones by themselves have not been clearly identified in humans as one of the factors responsible for preventing cancer. In addition, other bioactive compounds in soy foods, such as enzyme inhibitors or saponins, could be important in the process of cancer protection (Birt et al. 2001). To understand better if isoflavones by themselves are cancer protective, animal studies and in vitro data are of great interest.

#### b. Animal studies

A great number of studies performed in animals have investigated the possible beneficial effects that soyfoods and in particular isoflavones may have in protecting against cancer. Cancer was induced either by radiation or chemical injection to mimic a model that can be extrapolated to humans. For example, mammary cancer can be induced by radiation, N-methyl-nitrosourea (MNU) or 7,12 dimethylbenz[a]anthracene (DMBA). Barnes et al. (1990) found a dose response between exposure to soy protein isolate (20 to 200 g/kg diet) and mammary cancer (up to 50% reduction with 200g soy protein isolate/kg diet) in rats. More recently, Cohen et al. (2000) examined the effect of soy protein isolate (20 or 200 g/kg diet) with (2.89 mg total isoflavone/g isolate; 50% aglycone/50% glucosides) or without isoflavones with respect to mammary cancer development in F-344 rats (n=30/group). Twenty or 200 g SPI/kg diet provided ~105 and 215  $\mu\text{mol}$  total isoflavone/kg BW/d. Cancer was induced by N-methyl-nitrosourea (40 mg/kg body weight; animal age was 50d) one week after the feeding period started and continued for 18 weeks after cancer initiation. None of the treatment groups prevented cancer compared to the control fed casein (n=10), as observed in tumor incidence, multiplicity, volume and latency, thereby questioning the role of isoflavones and soy in general as being cancer-protective.

Without a soy protein matrix, isoflavones by themselves have also been studied, especially genistein. Its weak estrogenic or anti-estrogenic activity has been examined along

with its ability to inhibit protein tyrosine kinase and DNA topoisomerase, two enzymes necessary for proliferation of cancer cells (Messina et al. 1994). Two studies by Lamartiniere's team (Lamartiniere et al. 1995; Fritz et al. 1998) focused on breast cancer and genistein administered neonatally or perinatally to female Sprague-Dawley rats. When administered neonatally (Lamartiniere et al. 1995) at day 2, 4 and 6 with 5 mg genistein in 20  $\mu$ L DMSO subcutaneously (or 370  $\mu$ mol genistein/kg BW/d; control was DMSO alone) and cancer induced at d 50 with 80 mg DMBA/kg body weight, genistein-exposed animals had a significant delay in the appearance of tumors ( $124 \pm 33$  d) compared to the controls ( $87 \pm 37$  d). By d 190, only 88% of the genistein exposed animals developed tumors compared to 100% of the controls. Moreover, the mean number of tumors per animals was lower with the genistein treatment ( $3.67 \pm 0.45$ ) compared to the control group ( $6.35 \pm 0.67$ ). In this study, genistein had a role in protecting against cancer. However, the route of administration (subcutaneous and not oral) and the dose injected (pharmacologic, not dietary) were not suitable for extrapolation to humans (dose given on molar basis was  $\sim 100$  times higher than normal dietary dose in humans). Indeed, it would be unethical to inject intravenously or subcutaneously such large doses of isoflavone without knowing potential toxic or adverse effects that might occur. Moreover, these studies should be conducted so that they would be relevant to dietary ingestion of isoflavones by humans. This includes the process of metabolism/absorption/microbial degradation, which are essential factors when studying these compounds (See section C).

In a subsequent study (Fritz et al. 1998), animals were fed genistein at 0, 25 or 250 mg/kg diet (0, 8.8 and 92  $\mu$ mol/kg BW/d) from d 0 to d 21. At d 50, mammary cancer was initiated with DMBA and animals were fed a genistein free diet from d 21 to d 200. Animals fed the highest dose of genistein had a 50% reduction in the mean number of tumors compared to animals fed no genistein during the first 21 d of the experiment. ( $4.4 \pm 0.6$  vs.  $8.8 \pm 0.8$  tumors/animal). By d 50 and compare to the control, the group fed 92  $\mu$ mol/kg BW/d had fewer terminal end buds ( $65 \pm 6$  vs.  $31 \pm 4$  end buds/animal) and type I lobules ( $45 \pm 4$  vs.  $15 \pm 3$  lobules/animals). Terminal end buds and type I lobules were chosen in this study to reflect mammary gland differentiation, a phenomenon associated with increased

risks of breast cancer. The authors concluded that genistein had a long-term protective effect against mammary cancer.

This latter study was more relevant to humans because of oral ingestion of isoflavones at doses relevant but still higher than those of a typical Asian diet. On the other hand, complete extrapolation between the human and any animal model should take into account interspecies metabolic and physiologic differences. This might explain why a higher dose is required for animals with the authors' assumption that it is still relevant to humans (based on blood isoflavone concentration and previous bioavailability data in both human and animal models) (Fritz et al. 1998). Thus a dose of  $\sim 50$   $\mu\text{mol/kg}$  body weight/d in rats ( $\sim 10$ - $15$  fold higher than humans) would best mimic an Asian human diet (Fritz et al. 1998).

Prostate cancer also has been extensively studied. Pollard et al. (1997) used Lobund-Wistar rats fed soy protein isolate with high (33.8 mg total isoflavone/kg diet or  $\sim 12$   $\mu\text{mol/kg}$  BW/d) or low isoflavone (2.18 mg/kg diet or 0.8  $\mu\text{mol/kg}$  BW/d) dose. Control animals were fed casein. MNU (30 mg/kg body weight) was used as a prostate cancer inducer and testosterone propionate as a promoter (implanted 7 d after MNU injection). Two designs were applied for this study: diets were given to animals 27 d before or 7 d after MNU. Animals fed high isoflavone pre-MNU had a 27% increase delay in cancer development compared to low isoflavone pre-MNU rats (10 vs. 7.3 months). In addition, rats fed high isoflavone intake before MNU had a lower incidence of cancer compared to rats fed isoflavones post-MNU (8/24 vs. 18/35), but the time of cancer formation was the same in rats fed high or low isoflavone doses post-MNU (10.6 vs. 9.3 months). This study suggests that isoflavones were protective against cancer before it occurred but they were not as effective once initiation/promotion has started. Therefore, isoflavones may be considered as prostate cancer-preventive but not curative compounds.

Another model to study isoflavones and cancer is the use of transgenic animals capable of developing spontaneous tumors in a short period of time, which avoids injection of chemicals to promote cancer. With respect to breast cancer, Jin and MacDonalds (2002) studied the effect of isoflavones in mouse mammary tumor virus (MMTV)-neu mouse, which develops mammary tumors spontaneously due to overexpression of the ErbB-2/neu/HER oncogene (cancer gene activated in mammary cancer in both animals and humans). Starting

at 7 up to 34 weeks of age, these mice (n=18/group) were fed AIN-93G diets containing no isoflavones, 250 mg/kg genistein (111  $\mu\text{mol/kg BW/d}$ ), 250 mg/kg daidzein (118  $\mu\text{mol/kg BW/d}$ ) or Novasoy (equivalent to 250 mg/kg genistein). Latency of tumor development increased from 23 weeks of age for control up to 27 weeks of age for all three treatment groups ( $p<0.05$ ). However, by week 34, there was no difference in tumor incidence among all groups (~90-100% animals developed tumors). Thus, this study agreed with Pollard et al. (1997) that isoflavones were cancer-protective (increased latency of tumor development), but they did not stop metastasis once it had started.

Mentor-Marcel et al. (2001) used a transgenic mouse model of prostate cancer in mice (TRAMP model) to mimic prostate cancer. Starting at 5-6 weeks of age up to 28-30 weeks of age, these mice were fed genistein at 0, 100, 250 and 500 mg/kg diet (0, 44, 111 and 222  $\mu\text{mol/kg BW/d}$ ) (n=25, 10, 17 and 7, respectively). Not only was no toxic effect of genistein observed, but also a decrease in poorly differentiated adenocarcinoma was observed in a dose dependent manner at 0, 44 and 111  $\mu\text{mol/kg diet BW/d}$  (~30, 20 and 10% animals, respectively). The dose of 222  $\mu\text{mol/kg BW/d}$  was similar to 111  $\mu\text{mol/kg BW/d}$  in effect. On the other hand, proportions of well-differentiated and moderately differentiated prostatic adenocarcinoma were not affected by genistein feeding. The authors concluded that genistein had a significant protective effect of genistein on prostate cancer.

### c. *In vitro* studies

Studies on *in vitro* proliferation or inhibition of cancer cell lines by isoflavone were conducted to better understand the possible mechanisms of action of these compounds. As in animal studies, genistein has been extensively investigated because of its ability to inhibit several enzymes necessary for growth and proliferation of cancer cells (Messina et al. 1994). Mechanisms of action of daidzein and glycitein with respect to cancer are somewhat more obscure and have not been as well investigated as genistein in either animal or *in vitro* studies.

Hsieh et al. (1998) found that a low concentration of genistein (10 nM) enhanced proliferation of human breast cancer cells (MCF-7) while concentration above 20  $\mu\text{M}$

inhibited the growth ( $IC_{50}$  = 16-27  $\mu$ M). Compared with control, 1  $\mu$ M genistein produced a 3-fold greater cell proliferation, while 100  $\mu$ M genistein decreased cell proliferation by 40%. An increase in proliferation was accompanied by an increase in expression of the pS2 gene, which is estrogen-dependent and related to cell proliferation. Genistein had a dual effect and acted as an estrogen agonist at low concentrations and an antagonist at high concentrations. With another breast cancer cell line (MDA-468) that did not contain estrogen receptors, Peterson et al. (1991) found an inhibition of proliferation with a similar high dose of genistein, but a similar lower dose did not enhance proliferation. Thus, the presence of estrogen receptors may be required for genistein to activate cancer cells, while cellular inhibition may occur by estrogen receptor-independent mechanisms.

Akiyama et al. (1987) used epidermal growth factor receptors from cancer A-431 cells. These receptors are tyrosine-kinase dependent, enzymes necessary for the phosphorylation and activation of some cancer-associated genes. The rationale for using these cells was that the intricate relationship between EGF receptor, tyrosine kinase and cell growth proliferation created a suitable model for inhibition of cell growth by genistein, if the mechanisms involved inhibition of tyrosine kinase. Genistein inhibited tyrosine kinase in a dose dependent manner (0, 1, 3, 10, 30 and 100  $\mu$ g/mL; graphical data provided the lowest dose with an effect) confirming the hypothesized inhibitory effect. However, these results and conclusions were reported from observing blots without statistical analysis.

Another possible explanation of genistein's inhibitory effect on cancer cells is the inhibition of DNA topoisomerase and DNA cleavage. Salti et al. (2000) examined the effect of genistein in apoptosis and topoisomerase II-mediated DNA breakage in colon cancer cells (HT-29). Using fluorescent techniques to assess DNA breakage, they found that genistein increased DNA breakage in a dose-dependent manner at 10, 30, 100 and 200  $\mu$ M (DNA damage score from ~75 to ~150 arbitrary units one hour after treatment, extrapolated from graphical data). Apoptotic cells increased by  $54 \pm 3.5\%$  and  $94 \pm 1.4\%$  compared to untreated cells at 60 and 150  $\mu$ M genistein, respectively.

Vissac-Sabatier et al. (2003) focused on how phytoestrogen-rich diets impact expression of Brca1 and Brca2, two major tumor suppressor genes of human breast cancer. They analyzed the expression of these genes in mammary glands of ovariectomized (ovx) 90



d old Wistar rats fed different diets for 90 d (n=10/group; controls were sham operated and ovx animals fed the same diet without soy or isoflavones). Diets contained Novasoy (159, 156 and 33 mg of genistein, daidzein and glycitein/g Novasoy, respectively) at 20, 40 or 80 mg total isoflavone/kg body weight/day. Soylife contained 5.55, 15.36 and 14.09 mg of genistein, daidzein and glycitein/g Soylife, respectively and was given at dose of 4, 8 and 16 mg total isoflavone/kg body weight/day. None of the treatments increased expression of Brca1 while doses of 8 mg Soylife and 80 mg Novasoy increased Brca2 expression by a factor ~2 and ~3, respectively. In a separate part of the study, comparison of animals fed 40 and 80 mg isoflavone/kg body weight/day from Novasoy to animals treated with  $\alpha$ -estradiol (30  $\mu$ g/kg body weight) showed that all three groups increased significantly Brca1 levels compare to sham or ovx control animals. Brca2 levels were significantly increased with estradiol and 80 mg isoflavone from Novasoy/kg body weight/day animals. The authors concluded that isoflavones from supplements significantly increased the expression of cancer-suppressor genes. Different proportions of one isoflavone to the other was not a major factor in the outcome measured, thus suggesting that all three isoflavones may work in an additive manner to prevent cancer.

Therefore, isoflavones' mechanisms of action to prevent cancer are multiple and will deserve more investigation in animal and possibly human trials. On the other hand, most of the published studies on genistein and cancer-associated enzyme inhibition were done with pharmacological doses of genistein (greater than the highest dose achievable through a normal diet rich in soy products). This calls into question of extrapolating the data to in vivo situations (Barnes et al. 1995).

#### d. Summary

Isoflavones as cancer-protective agents are under investigation. Despite epidemiological data that showed a correlation between consumption of soy and/or isoflavone and lower risk of cancer (Hirayama. 1979; Zheng et al. 1999), animal and in vitro studies provided mixed conclusions, especially with respect to the doses necessary to inhibit cancer (above dietary levels; Lamartiniere et al. 1995), the apparent dual action of genistein

(Hsieh et al. 1998) and the actual modes of action observed in vitro that may not be valid in vivo (Akiyama et al. 1987; Markovits et al. 1989; Constantinou et al. 1996). Estrogen-dependent cancers, such as breast cancer, may be studied separately from estrogen-independent cancers. As seen in the toxicity section (McMichael-Phillips et al. 1998), there also may be a dual action of genistein in humans, where it is inhibitory when administered before but stimulatory after cancer development. This would support the conclusion that isoflavones might be cancer-protective, but not curative.

## 2. Isoflavones and blood lipid profile

Among all the health-enhancing or protecting effects that have been hypothesized for isoflavones, the cholesterol-lowering effect has been one of the most controversial. Many studies have been performed in both animals and humans, but the data have led to some discrepancies and opposing opinions as to how effective isoflavones are in improving the blood lipid profile.

### a. Human studies

Human studies focusing on the hypocholesterolemic effects of isoflavones have also given rise to variable results. Several factors may be considered when analyzing the data: age, baseline blood lipid profile, isoflavone source (purified, dietary supplement or part of soy protein), dose of isoflavones and duration of the feeding period (Demonty et al. 2003). Great variations of these factors from one study to the next make comparative analysis of the data a real challenge. According to Goodman-Gruen et al. (2001), one of the major disadvantages of providing soy protein when focusing on isoflavones and cholesterol is the variety of other bioactive compounds present, such as saponins that might interfere with the hypothesized isoflavone cholesterol-lowering effect. It also seems that providing isoflavones to individuals with moderate to severe baseline hypercholesterolemia might result in more improvements compared with individuals who have normal baseline blood lipid profiles as seen in obese v. lean rats (Peluso et al. 2000).

An epidemiological study performed by de Kleijn et al. (2002) grouped 939 postmenopausal women into quartiles according to their isoflavone intake. They found that women in the highest quartile of isoflavone intake ( $>0.9 \mu\text{mol/kg BW/d}$ ) had significantly lower plasma triglyceride ( $1.46$  vs.  $1.62 \text{ mmol/L}$ ) and mean cardiovascular risk factor metabolic score ( $1.30$  vs.  $1.74$  score, calculated based on systolic and diastolic blood pressure, plasma triglycerides and HDL-C, body mass index and waist-to-hip ratio values for each subject) compared to the lowest quartile of isoflavone intake ( $<0.3 \mu\text{mol/kg BW/d}$ ), respectively. Also comparing postmenopausal women ( $n=208$ ) based on their isoflavone consumption, Goodman-Gruen et al. (2001) found that the group with the highest intake of genistein ( $>3.7 \mu\text{mol/kg BW/d}$ ) had significantly lower BMI ( $24.5 \pm 3.5$  vs.  $26.2 \pm 4.4$ ) and greater HDL-C ( $1.5 \pm 0.04$  vs.  $1.4 \pm 0.65 \text{ mmol/L}$ ) compared to the group with no intake of genistein. Another epidemiological study (Ho et al. 2000) done in both Chinese men ( $n=500$ ) and women ( $n=510$ ) showed that higher soy and isoflavone consumption was negatively correlated with total cholesterol ( $R=-0.225$ ;  $p=0.046$  and  $R=-0.283$ ;  $p=0.033$ ) for men and women less than 50 years of age, respectively. Isoflavone consumption also correlated negatively with LDL-C for men ( $R=-0.203$ ;  $p=0.026$ ) and women  $< 50$  ( $R=-0.242$ ;  $p=0.043$ ). Women older than 50 years did not show any correlation between blood lipid profile and isoflavone intake, because of significantly lower dietary intake of soy and isoflavone compared to men ( $66.3 \pm 71.68 \text{ mg/wk}$  vs.  $101.6 \pm 107.5 \text{ mg/wk}$ ) and women less than 50 years of age ( $66.3 \pm 71.68 \text{ mg/wk}$  vs.  $82.4 \pm 98.0 \text{ mg/wk}$ ). For this study, exclusion criteria were not listed, but all subjects that received dietary assessment at the hospital were eligible up to  $n=100$  per age group ( $<34$ ,  $35-44$ ,  $45-54$  and  $>55$  years of age). Confounding factors included in these analyses were sociodemographic variables of sex, age, marital status and level of education.

These epidemiological studies have led researcher to conclude that isoflavone consumption had a positive impact on blood lipid profile in both men and women and that there may be a minimum threshold intake required to observe an effect. However, clinical trials have showed much more varied and controversial results.

Crouse et al. (1999) studied the effect of isoflavones in moderately hypercholesterolemic men and women ( $n=156$ ; LDL cholesterol concentrations from  $3.62$  to

5.17 mmol/L). Subjects were divided into 5 groups (n=31) and given beverages containing either 25 g casein/day, 25 g ethanol-washed isolated soy protein/day that contained 3mg total isoflavones (0.15  $\mu\text{mol/kg BW/d}$ ) or 25 g soy protein/day at three levels of isoflavones: 27, 37 or 62 mg total isoflavones (1.4, 1.95 and 3.3  $\mu\text{mol/kg BW/d}$ ) for 9 weeks. Subjects given 3.3  $\mu\text{mol/kg BW/d}$  had significantly lower levels of total (4% reduction) and LDL cholesterol (6% reduction) compared with casein controls. In addition, when subjects were divided in two groups (same n) based on their baseline cholesterol values, subjects with baseline LDL in the highest half ( $>4.24$  mmol/L) had an even greater decrease in total (9%) and LDL cholesterol (10%) compare to casein. An 8% decrease was also observed in total and LDL-cholesterol of baseline-high LDL subjects fed 1.95  $\mu\text{mol/kg BW/d}$  compare to casein group. Thus, this study showed not only a threshold effect of isoflavones in order to improve blood lipid profile but also that more severely hypercholesterolemic subjects had greater benefit from isoflavone than did moderately hypercholesterolemic subjects.

An interesting aspect of cholesterol metabolism in pre-menopausal women was investigated by Merz-Demlow et al. (2000). They found that the baseline blood lipid profile differed in the different phases of the menstrual cycle (early follicular, midfollicular, periovulatory and midluteal phases). Consequently, they used a cross-over study to investigate the effect of no ( $<10$  mg; 0.5  $\mu\text{mol/kg BW/d}$ ), low ( $\sim 65$  mg; 3.4  $\mu\text{mol/kg BW/d}$ ) and high ( $\sim 130$  mg/d; 6.8  $\mu\text{mol/kg BW/d}$ ) isoflavones diet from soy protein isolate, each applied during a full menstrual cycle (n=13). Women given the low isoflavone dose did not differ from the controls (no isoflavone) in any blood lipid value for any menstrual cycle phases. However, women fed the high isoflavone diet showed significantly lower LDL-C compare to control in the midfollicular ( $2.20 \pm 0.04$  vs.  $2.38 \pm 0.04$  mmol/L) and periovulatory ( $2.07 \pm 0.05$  vs.  $2.3 \pm 0.05$  mmol/L) phase. Also in the periovulatory phase, the high isoflavone diet improved the total:HDL-C ratio ( $2.91 \pm 0.06$  vs.  $3.24 \pm 0.05$ ) and LDL-C:HDL-C ratio ( $1.69 \pm 0.05$  vs.  $1.96 \pm 0.04$ ) compare to control. Regardless of the menstrual phase, it seemed that a 3.4  $\mu\text{mol/kg BW/d}$  dose of isoflavones was not sufficient to improve the blood lipid profile in pre-menopausal women, while a dose of 6.8  $\mu\text{mol/kg BW/d}$  was effective. The same results were obtained in hypercholesterolemic postmenopausal women (n=18; time of study=93 d, crossover design with the same doses of isoflavones applied) by

Wangen et al. (2001). LDL-C cholesterol was significantly lower during high isoflavone intake compared to control ( $3.01 \pm 0.05$  vs.  $3.22 \pm 0.05$  mmol/L). The low isoflavone feeding gave an intermediate result that was not different from control or high isoflavone ( $3.05 \pm 0.05$  mmol/L). While a dose of  $3.3 \mu\text{mol/kg BW/d}$  was enough to produce significant results in Crouse et al. (1999) study (men and women), it seemed that in women (pre- or postmenopausal), a higher threshold isoflavone dose ( $\sim 6.8 \mu\text{mol/kg BW/d}$ ) was needed to obtain significant results (Merz-Demlow et al. (2000); Wangen et al. (2001)). Therefore, men and women may differ in the minimum dose required to obtain significant improvement in blood lipid profile. This could be partly explained by the fact that men and women differ in baseline blood lipid profile values. Freedman et al. (2004) compared plasma lipoprotein levels in men and women as part of the analysis of the Framingham study. They found that, when adjusted for age, men had significantly higher concentrations of triglycerides (mean  $0.24 \text{ g/L}$  difference between men and women;  $p < 0.001$ ) and LDL-cholesterol (mean  $0.06 \text{ g/L}$  difference;  $p < 0.001$ ) while women had significantly higher HDL-C (mean  $0.12 \text{ g/L}$  difference;  $p < 0.001$ ). Thus, women have lipid profiles less prone to cardiovascular disease, so that a higher dose of isoflavones is required to exert a significant impact on their blood lipid profile.

Baseline cholesterolemia is also a crucial factor when designing this type of study. To accentuate the controversy surrounding the possible role of isoflavone in decreasing cholesterol, several studies have concluded that isoflavones do not improve the blood lipid profile. For example, Jenkins et al. (2002) examined the effect of a control (no soy products), high ( $73 \text{ mg}$ ;  $3.8 \mu\text{mol/kg BW/d}$ ) and low ( $10 \text{ mg/d}$ ;  $0.8 \mu\text{mol/kg BW/d}$ ) isoflavone containing soy protein diet (cross-over design; each diet=1 month) involving 41 hypercholesterolemic men and post-menopausal women. Guidelines to design the diets were followed from the National Cholesterol Education Program Step II. Regardless of the isoflavone dose, soy protein significantly decreased LDL-C, improved the total:HDL cholesterol ratio, the LDL:HDL cholesterol ratio and coronary artery disease risk. This study demonstrated that soy protein improved blood lipid profile but the role of isoflavone in this mechanism was not clearly established, probably due to some design-related issues. No

group was provided any soy protein, nor were groups given isoflavone without a soy protein matrix included, thus producing controversial conclusions.

Isoflavone without a soy protein matrix was also investigated. Dewell et al. (2002) provided 36 moderately hypercholesterolemic post-menopausal women (mean total cholesterol =  $6.6 \pm 1.3$  mmol/L) with 150mg isoflavone supplement/day ( $7.9 \mu\text{mol/kg BW/d}$ ;  $n=20$ ) or placebo ( $n=16$ ) for 2 months. Isoflavone tablets contained 90% aglycones/10% glucosides; analysis of other bioactive components found in the supplement was not performed. No significant differences were found between baseline and two months for the isoflavone fed group in total cholesterol and HDL-cholesterol. No differences were found in total cholesterol and HDL cholesterol between placebo and isoflavone groups. It is possible that in this study, despite a dose that should have been high enough to produce an effect, baseline moderate hypercholesterolemia may not be high enough to observe an effect as seen by Crouse et al. (1999).

Similar results to Dewell et al. (2002) were found by Hodgson et al. (1998) with 46 men and 13 postmenopausal women (all normocholesterolemic) given either a placebo or isoflavone (55 mg;  $2.9 \mu\text{mol/kg BW/d}$ ) tablets (analysis of other bioactive components found in the supplement was not performed) for one month, after adjustment for age, gender, weight or urinary excretion of isoflavones. In this case, the dose of isoflavones applied along with normal baseline cholesterol levels might have led to non-significant effects.

To clarify the dose necessary for isoflavones to decrease LDL-C, Zhuo et al. (2004) performed a meta-analysis of 8 human studies with no, low and high isoflavone-containing soy protein treatments. They concluded that a minimum dose of 50 g soy protein (96 mg isoflavone/d;  $\sim 5 \mu\text{mol/kg BW/d}$ ) was required to observe an effect. Both normo- and hypercholesterolemic subjects showed significant decreases LDL-C with 96 mg isoflavone (0.14 and 0.18 mmol/L decrease, respectively). However, those with baseline hypercholesterolemia showed an even greater p value (0.0008) in decreasing LDL-C compared to those with baseline normocholesterolemia ( $p=0.03$ ).

## b. Animal studies

Animal studies on soy protein and isoflavones with respect to cholesterol-lowering effects have been investigated in a few animal models. Golden Syrian hamsters have been one of the most extensively studied because cholesterol metabolism in Golden Syrian hamsters closely resembles that of humans and hypercholesterolemia can be induced in a short period of time in this animal model (Bravo et al, 1994).

Song et al. (2003) fed male and female Golden Syrian hamsters (n=10/sex/group; 6-8 weeks of age) a high fat diet containing 130  $\mu\text{mol/kg BW/d}$  of purified daidzein for 10 weeks. Total cholesterol was significantly reduced by ~24% and non-HDL cholesterol by ~35% in both sexes. The fact that only daidzein was fed in its purified form make extrapolation to humans difficult considering that dietary isoflavones are consumed in a food matrix with different proportions of each isoflavone.

Lin et al. (2004) fed male hamsters (n=20/groups; 5 weeks of age) for 5 weeks 30% fat, 0.08% cholesterol Western-type diets containing either casein, plant sterol esters, soy isoflavones from Novasoy® and different combination of these four diet components. Hamsters fed isoflavones (~80  $\mu\text{mol/kg BW/d}$ ) did not differ from the casein control for total cholesterol, triglycerides or HDL-cholesterol ( $p>0.05$ ). This study disagrees with Song et al. (2003), but major differences in study design make comparisons almost impossible. Song et al. (2003) fed both sexes at a higher dose/kg diet of purified daidzein (130  $\mu\text{mol/kg BW/d}$ ), while Lin et al. (2004) used only male fed Novasoy at a dose of 80  $\mu\text{mol/kg BW/d}$ . Therefore, both sexes should be included for such a design and a minimal isoflavone dose expected to produce significant results should be considered as well.

Blair et al. (2002) examined male (n=75) and female (n=74) Golden Syrian F1B hybrid hamsters (12 weeks of age) fed either casein, soy protein with (450 mg isoflavone/kg diet or 166  $\mu\text{mol/kg BW/d}$ ) or without (10 mg total isoflavone/kg diet or 3.7  $\mu\text{mol/kg BW/d}$ ) isoflavone, isoflavone-deprived soy protein and casein to which an isoflavone supplement (325 mg total isoflavone/kg diet or 120  $\mu\text{mol/kg BW/d}$ ) was added. All diets were extrapolated in composition to mimic a typical Western diet (high saturated fat from lard, beef tallow, and butter and high sugar from sucrose). After 16 weeks, none of the treatments

lowered LDL+VLDL-cholesterol (C) in females whereas in males, the three soy protein-based diets, but not the casein+isoflavone diet reduced LDL+VLDL-C significantly (~25 to 50%). Plasma LDL+VLDL-C was positively correlated with plasma isoflavone in females ( $r=0.65$ ;  $p=0.02$ ) but not in males ( $r=0.47$ ;  $p=0.28$ ). HDL-C was significantly higher in females fed soy protein with or without isoflavone (~25%) while males did not show any improvement in HDL-C after soy feeding. This study showed a clear gender-related effect associated with soy protein, but isoflavone as part of soy protein or supplement did not show any positive effect in improving blood lipid profile. On the other hand, animals in Blair et al. (2002) were older than in the Lin et al. (2004) and Song et al. (2003) studies. Differences in background diet composition may also alter the outcome. For example, the main source of saturated fat in Song's study was coconut oil while in Blair's study, lard, beef tallow and butter were used. This may have had an influence in raising cholesterol levels in the animals so that induction of hypercholesterolemia and choices of diets were important factors to consider when designing such a study. Control diets may have also contributed to the results obtained. While Blair et al. (2002) used a control diet where soy protein was replaced by casein, Song et al. (2003) used the same diet as the treatment without isoflavone. Therefore, a control diet closer to that of the treatment may be more appropriate than a control diet with changes in major nutritional factors, such as the protein source.

Using a rat model, Peluso et al. (2000) examined the effect of isoflavone in male obese Zucker rats and male lean Sprague-Dawley rats. Diets were casein, low isoflavone soy protein (38 mg/kg diet or 14  $\mu\text{mol/kg BW/d}$ ) or high isoflavone soy protein (578 mg/kg diet or 214  $\mu\text{mol/kg BW/d}$ ). All diets were formulated to contain 18.6% protein, 69.4% carbohydrate and 12.0% fat. Plasma and liver total cholesterol was significantly reduced in rats fed both soy protein diets (21 and 29% for low and high isoflavone doses, respectively) compared with casein fed control obese animals. Liver total triglyceride level, liver cholesteryl ester and liver weight were significantly lower in high vs. low isoflavone diet ( $p<0.05$ ; numerical data not available), which indicated a possible greater effect on cholesterol metabolism when isoflavones were present. In lean animals, no treatment diets improved plasma total cholesterol and triglyceride. However, feeding a high isoflavones diet significantly decreased liver total cholesterol and cholesteryl ester levels compare to casein



feeding but not low isoflavones feeding. This study produced mixed conclusions about the role of isoflavone in cholesterol metabolism. However, it seemed that results were more significant in obese than lean animals, suggesting that baseline cholesterol profile is an important factor to consider. Higher starting total cholesterol concentrations may be necessary for isoflavones to exert a significant effect compared to a more “healthy” baseline blood lipid profile.

### c. Mechanisms of cholesterol lowering by isoflavones

Despite conflicting results of isoflavone on cholesterol metabolism, a few studies have focused on the possible mechanisms by which isoflavones interact with lipoproteins. One possible mechanism is a decrease in oxidation of LDL, which decreases uptake by macrophages in the arterial wall, thereby leading to a decrease in plaque formation. This mechanism may not be a cholesterol-lowering effect per se, but cardiovascular disease and blood lipid profile are highly correlated with each other. Jenkins et al. (2000) looked at the effect of a low-fat diet vs. a low-fat diet + 33g soy protein (86 mg isoflavone/d; 4.5  $\mu\text{mol/kg}$  BW/d) fed for 1 month to 31 hyperlipidemic subjects. Mean total cholesterol and LDL-cholesterol decreased significantly between control and soy diets ( $-0.38 \pm 0.07$  mmol/L and  $-0.27 \pm 0.07$  mmol/L decrease for total and HDL-C, respectively). In addition, *ex vivo* oxidized LDL, expressed as conjugated dienes ( $-7 \pm 2$  pmol/L) and conjugated dienes:LDL ratio ( $-0.7 \pm 0.4$ ) decreased significantly from control to soy treatment. The author concluded that isoflavone-rich foods improve blood lipid profiles, partly because of a decrease in LDL oxidation. However, a soy treatment without isoflavones was not included in this study, so that the actual role of isoflavones vs. other bioactive components found in soy protein in decreasing LDL oxidation and improving blood lipid profiles has not been fully proved. In a cross-over design of 2 menstrual cycles with placebo and 2 menstrual cycles of red clover tablets supplementation (43 mg/d; 2.2  $\mu\text{mol/kg}$  BW/d), Samman et al. (1999) did not find any significant changes in blood lipid profile or in *ex vivo* copper-induced LDL oxidation (lag time of  $32.9 \pm 3.1$  vs.  $30.4 \pm 2.9$  min for placebo and supplement, respectively) from plasma sample obtained from the subjects (14 premenopausal women) in either study design.

Therefore, isoflavone without a protein matrix did not improve LDL oxidation but the dose of supplementation may have been below a threshold level.

LDL uptake by the liver is due to a higher presence of LDL receptors, thereby reducing LDL-C. Baum et al. (1998) studied for 24 weeks 66 hypercholesterolemic postmenopausal women fed soy protein containing either 56 mg or 90 mg total isoflavones/d (2.9 and 4.7  $\mu\text{mol/kg BW/d}$ ) and a control group fed a casein-based diet. Both doses of isoflavones increased significantly expression of LDL receptor ( $127 \pm 17.7$  and  $175 \pm 31\%$  of baseline measurements for 2.9 and 4.7  $\mu\text{mol/kg BW/d}$  groups, respectively), as reflected by a higher mononuclear cell LDL receptor mRNA concentrations compared to the control group ( $74 \pm 7.8\%$  of baseline). Also focusing on LDL receptor, Kirk et al. (1998) used mice that lacked LDL-receptor (LDL-null) and wild type C57BL/6 mice, each type fed a diet containing either high ( $\sim 350$  mg total isoflavone/kg diet; 155  $\mu\text{mol/kg BW/d}$ ) or low ( $\sim 35$  mg/kg diet; 15  $\mu\text{mol/kg BW/d}$ ) levels of isoflavones for 6 weeks. Plasma cholesterol and LDL oxidation were similar between the two diets in the LDL-null animals, whereas in wild type mice, high isoflavones decreased total cholesterol by 30% and atherosclerotic lesions by 50% compared to the low isoflavone diet. This study not only showed a significant effect of isoflavones on cholesterol metabolism, but also the important relationship with LDL receptors in achieving these effects. A greater increase in LDL receptor expression in the highest compared to the lowest dose of isoflavones in wild type mice also suggested the possibility of a dose-response up-regulation effect. However, regression and correlation analysis to test this hypothesis were not performed in that study and deserved further investigations.

#### d. Summary

From the studies selected throughout this review, isoflavones may have a positive action on blood cholesterol. The most important factors to consider are gender, dose ( $\sim 100$  mg isoflavone/d or 5.3  $\mu\text{mol/kg BW/d}$ ), baseline cholesterol (hypercholesterolemia) and time (1 month minimum) in order to obtain significant improvement in blood lipid profiles. With respect to animal models, uncertainty factors between humans and animals and higher doses

(~10 to 15 folds) required to observe similar effects (as seen in cancer section) would lead to the hypothesis that if a dose of ~5  $\mu\text{mol/kg BW/d}$  is required to observe an effect in humans with relevance to dietary intake, a dose of ~ 50-75  $\mu\text{mol/kg BW/d}$  applied to animals producing significant results would be considered for extrapolation to humans. Higher doses would be classified as pharmacological (use of supplements; dose not achievable with diet only) and results observed deserve further investigation to ameliorate relevance to humans.

### 3. Isoflavones and immune function

Among all of the possible health-related effects that may be associated with isoflavone, enhancement of immune function has been demonstrated, but the number of studies published is somewhat more limited compared to other health effects. Zhang et al. (1999a) compared isoflavone aglycones and glucuronides, the two main forms found in plasma after ingestion, with respect to modulation of human natural killer cells. Concentrations of 0.1 to 10  $\mu\text{M}$  of daidzein and genistein glucuronides activated NK cells *in vitro* by ~30% and toxicity was not observed with isoflavone glucuronide concentration up to 50  $\mu\text{M}$ . Genistein (aglycone) activated NK cells at 0.1 to 5  $\mu\text{M}$  but inhibition was observed at 10  $\mu\text{M}$ . Consequently, isoflavones glucuronides were as efficient, but less toxic than their aglycone equivalent in benefiting NK activity. Guo et al. (2001) used an *in vivo* model to study isoflavone and immune function. B6C3F1 mice were gavaged for 28 d with different doses of genistein (0, 2, 6 or 20 mg/kg body weight; 0, 7.4, 22 or 74  $\mu\text{mol/kg BW/d}$ ;  $n=12/\text{group}$ ), injected with  $10^5$  B16F10 melanoma cells 24h after the last dose of genistein and killed 18 d after cancer cells injection. Host resistance to cancer formation and cytotoxic T cells activity were proportional to the dose of genistein fed. After killing, *in vitro* basal NK activity was not affected by genistein while IL-2-stimulated NK activity was significantly increased in animals originally fed 20 mg genistein/kg body weight (effector:target ratio was 25:1). Splenic B-cell percentage and IgM and IgG antibody cell response were not affected by genistein. This study showed that genistein was tumor protective, probably due to an increase in immune function and resistance. Guo et al. (2002) also studied the effect of genistein intake in a two-generation rat model. Sprague-Dawley rats (F0) were fed three

doses of genistein: low (25 ppm; 9  $\mu\text{mol/kg BW/d}$ ), medium (250 ppm; 92  $\mu\text{mol/kg BW/d}$ ) or high (1250 ppm; 462  $\mu\text{mol/kg BW/d}$ ) starting at d 7 of pregnancy to postpartum d 51. Control animals received a diet deprived of any isoflavone. F1 animals (males and females) received the same genistein doses as their mothers up to 64 d after birth. F0 animals did not have any significant changes in proportions or amounts of T-cells, B-cells or NK cells. NK activity was significantly higher in the medium (111% increase) and high group (133% increase). In F1 male rats, significant increases in numbers of splenocytes, T cells, T-helper cells and cytotoxic T-cells were observed at all 3 doses of genistein. NK cell number was unaffected but the proportion of NK to other types of splenic cells was significantly lower than the control at all 3 doses. NK activity showed a significant increase at 462  $\mu\text{mol/kg BW/d}$  genistein with 200:1 effector:target ratio only. F1 female animals also showed increases in T cells numbers (462  $\mu\text{mol/kg BW/d}$ ), T helper cells (9 and 462  $\mu\text{mol/kg BW/d}$ ) and cytotoxic T cells (92 and 462  $\mu\text{mol/kg BW/d}$  1250ppm). Decrease in % NK cells was observed for 9 and 462 $\mu\text{mol/kg BW/d}$  genistein. Splenocyte IgM antibody-forming cell response to T-dependent antigen sheep RBC was increased by 122% at 462  $\mu\text{mol/kg BW/d}$ , while no significant data were found for the male F1 animals. Lastly, NK activity was enhanced only at 462  $\mu\text{mol/kg BW/d}$  with 100:1 effector:target ratio. The authors concluded that genistein did not have similar effects in F0 compared to F1 animals. In addition, male and female F1 rats did not produce the same results, suggesting that immunomodulation by isoflavone may have a gender-specificity, although the authors mentioned that the underlying mechanisms to explain these differences are still unknown (Guo et al. 2002). Different baseline immune functions and profiles, along with hormonal modulation of immune response probably specific to each gender could be hypothesized to be partly responsible for the observed data. Effects of isoflavones in vivo, particularly in humans, need further investigation. The possible relationships between isoflavones, tumor formation and immune system also deserve further investigations.

#### 4. Isoflavones and osteoporosis

Osteoporosis is defined as a “disease characterized by low bone mass and microarchitectural deterioration of bone tissue leading to enhanced bone fragility and a consequent increase in fracture incidence” (Melton and Riggs. 1983). This disease affects 35 million humans around the world and is more prevalent in women than men, especially after menopause which causes a lack of estrogen, leading to a decrease in bone formation and an increase in bone resorption (Melton et al. 1992). While drugs, such as raloxifene have a positive effect in preventing bone loss because of its weak estrogenic effect that partially replaces the loss of estrogen after menopause (Delmas et al. 1997), other estrogen-like dietary compounds, such as isoflavones contained in soy products, have been studied as osteoporosis-preventing compounds.

##### a. Human studies

Fewer studies focusing on isoflavones and osteoporosis have been performed in humans than in animals. Dalais et al. (1998) performed a double-blind, randomized, entry-exit, cross-over study in which they provided postmenopausal women (n=44) different diets for 12 weeks, containing either 45 g soybean grits (53 mg total isoflavone; 2.8  $\mu\text{mol/kg}$  BW/d) or 45 g wheat. Bone mineral content increased by 5.2% ( $p<0.04$ ) and 3.8% (non significant) for the soy and wheat diet, respectively compared to baseline. No changes were detected in bone mineral density. Dalais and colleagues concluded that isoflavones may have an impact in improving bone health in postmenopausal women, but further investigation would be needed.

Also in 1998, Potter et al. compared the effect of 2 doses of isoflavones using isolated soy protein (40 g/day for 6 months) as part of a parallel-group, double-blind trial in postmenopausal women. The two doses were 55 and 90 mg total isoflavones/d (2.9 and 4.7  $\mu\text{mol/kg}$  BW/d). Another group received casein as a control. The increase in lumbar spine bone mineral density and bone mineral content between baseline and 24 weeks was significantly greater in the 4.7  $\mu\text{mol/kg}$  BW/d group ( $0.892 \pm 0.114$  vs.  $0.912 \pm 0.119$  g/cm<sup>2</sup>

and  $49.6 \pm 8.3$  vs.  $50.8 \pm 8.7$  g/cm<sup>2</sup> at time 0 and 24 weeks for bone mineral density and bone mineral content, respectively) compare to casein ( $0.940 \pm 0.159$  vs.  $0.934 \pm 0.153$  g/cm<sup>2</sup> and  $56.0 \pm 11.0$  vs.  $55.4 \pm 10.3$  g/cm<sup>2</sup> at time 0 and 24 weeks for bone mineral density and bone mineral content, respectively), while the group receiving 2.9  $\mu$ mol/kg BW/d did not show significantly improved bone density and mineral content compare to casein. While this study suggested a minimal daily dose required for isoflavone to exert an effect on bone, interpretation of the data is somewhat confusing, considering that baseline values were different among groups and not taken into account in the analysis. Since there was no difference between time 0 and 24 weeks for each group, this study does not clearly demonstrate an effect of isoflavones on bone density.

Another study published by Alekel et al. (2000) focused on 69 perimenopausal women given 40 g of soy protein isolate with isoflavones SPI+ (80.4 mg total isoflavones/day or 4.2  $\mu$ mol/kg BW/d; n=24) or with reduced isoflavone content SPI- (4.4 mg/day; n=24). Another group (n=21) was given whey control protein (40 g/d). Both SPI+ and SPI- groups maintained their bone mineral density and content, while significant decreases were observed for both in the control group. Analysis of covariance showed that there was a treatment effect on bone mineral content. Multiple regression taking into account baseline values as well as other confounding factors, like body weight, showed that only SPI+ treatment contributed positively from the control for bone mineral density (5.6%;  $p=0.023$ ) and bone mineral content (10.1%;  $p=0.0032$ ). This study clearly showed that soy protein without isoflavones did not fully prevent bone loss, thereby demonstrating that isoflavones exerted an effect on bone density.

Despite the fact that these studies are over a short-term period (<1 year), they do provide some basis for future investigations that would either confirm or refute the potential role of isoflavones in preventing bone loss. On the other hand, the study of Ho et al. (2001) was conducted over 3 years with 132 women (30-40 yoa). Measurements at baseline and at 3 years included bone mineral density using DXA, soy food intake and other key nutrients, such as calcium. Consumption of isoflavones was divided in quartiles (0 to 0.15  $\mu$ mol/kg BW/d, n=37; 0.15 to 0.23  $\mu$ mol/kg BW/d, n=21; 0.23 to 0.39  $\mu$ mol/kg BW/d, n=29; 0.39 to 2.5  $\mu$ mol/kg BW/d, n=29) and the loss of bone mineral density was significantly less in the

highest quartile of intake (1.1%) compare to the lowest (3.5%), when confounding factors were included in the regression analysis. Overall, isoflavones accounted for 24% of the variance of the bone density regression slope obtained with individual values at time 0 and 3 years. This long-term study with sufficient power provided evidence that soy foods containing isoflavones help prevent bone loss in 30 to 40 years old women. However, subjects selected were not postmenopausal, which could lead to conduct additional studies with respect to the design of Ho et al (2001). It would be very instructive to follow premenopausal women who consume soy through menopause to determine the effects

#### b. Animal studies

Animal studies focusing on soy and osteoporosis were mostly performed as short-term studies with ovariectomized (ovx) animals, a procedure that is arguably relevant to menopause in women (Thompson et al. 1995).

Using ovariectomy to mimic menopause in 95 d-old Sprague Dawley rats (n=8/group), Arjmandi et al. (1996) found that ovx animals fed soy protein isolate (SPI) (227 g/kg diet; isoflavone content not mentioned) for 30d had significantly higher mean bone density of the right femur ( $\sim 1.5$  vs.  $\sim 1.3$  g/cm<sup>3</sup> bone volume;  $p < 0.001$ ) and the fourth lumbar vertebra ( $\sim 1.45$  vs  $\sim 1.3$  g/cm<sup>3</sup> bone volume;  $p < 0.05$ ) compared to ovx animals fed casein. The ovx group fed casein also had significantly greater serum concentrations of 1,25-dihydroxy-vitamin D, a marker of bone resorption, compared to animals fed SPI. Arjmandi and colleagues concluded that soy is effective in preventing bone loss due to ovarian hormone deficiency. However, they could not conclude if it was soy or isoflavones themselves that were responsible for the positive effect observed on bone density.

To understand the specific role that isoflavones may play in bone density, Arjmandi et al. (1998) conducted another study in which 48 Sprague-Dawley rats (90 d-old) were divided in 4 groups: 1 sham and 1 ovx group fed casein; 2 ovx groups fed a diet with SPI containing either normal (+; 473 mg total isoflavones as aglycone/kg diet) or reduced (-; 45 mg/kg diet) isoflavone content. The different diets were given for 35 d after surgery. Serum alkaline phosphatase, a nonspecific marker of bone formation, was significantly higher in the

SOY+ group ( $84 \pm 16.2$  U/L) compared to sham animals ( $106 \pm 12.6$  U/L). In addition, SOY+ animals had a mean right femur density ( $1.497 \pm 0.030$  g/cm<sup>3</sup> bone vol) similar to the sham group ( $1.522 \pm 0.041$ ), but significantly higher than ovx ( $1.449 \pm 0.044$ ) or soy- ( $1.452 \pm 0.030$ ) groups, which indicated that isoflavones prevented bone loss after ovx.

To understand which isoflavones play a role in bone density, Picherit et al. (2000) compared the effect of daidzein and genistein on ovx 12 month old female Wistar rats. Controls included sham operated and ovx animals fed a soy-free diet with no isoflavone supplementation (n= 13/groups; feeding period= 90 days). Daidzein and genistein were given orally in the diet at a dose of 10 mg or 37  $\mu$ mol/kg BW/d. They found that bone mineral density of lumbar vertebrae, femur and its metaphyseal (cancellous bone) and diaphyseal (cortical bone) zones analyzed by DEXA were not different between the daidzein and sham groups, while genistein and sham groups were not significantly different in the diaphyseal bone mineral density only (numerical data not available). No difference in bone turnover markers was observed between the groups. Overall, Picherit and colleagues concluded that daidzein was more efficient in preventing bone loss than genistein. This study was also of great interest because isoflavones were fed as pure compounds without a soy protein matrix, which clearly established the importance of isoflavones in preventing bone loss in the rat model. Another study from the same group published in 2001 looked at a dose response in the same animal model. OvX Wistar rats (10/groups) were fed 0, 20, 40 or 80 mg isoflavones (0, 74, 148, 296  $\mu$ mol/kg BW/d from Novasoy®) for 91 days. Ten sham-operated animals fed no isoflavones served as a control. Total and diaphyseal bone mineral density of the femur was not significantly different between sham controls and rats fed 74, 148 and 296  $\mu$ mol/kg BW/d whereas the rats fed 148 and 296  $\mu$ mol/kg BW/d had non significantly different metaphyseal femoral bone mineral density compared to sham operated animals and compared to each other. Urinary pyroxidinoline, a marker of bone resorption was also similar between sham, 148 and 296  $\mu$ mol/kg BW/d groups while significantly lower in the 74  $\mu$ mol/kg BW/d group (numerical data not available). On the other hand, rats fed 296  $\mu$ mol/kg BW/d showed a somewhat toxic effect with a significantly higher mean uterine weight ( $0.40 \pm 0.04$ g) compared to the other isoflavone doses ( $0.18 \pm 0.01$ ,  $0.19 \pm 0.02$  and  $0.22 \pm 0.03$ g for the rats fed 0, 74 and 148  $\mu$ mol/kg BW/d, respectively). Therefore, the optimal dose of



isoflavones to prevent bone loss without noticeable adverse effects has been established at 148  $\mu\text{mol/kg BW/d}$  in the ovx Wistar rat model. In humans, with an uncertainty factor of 10-15 described in the cancer section, a dose of efficacy in humans to exert significant bone-sparing effect should be between 15 and 20  $\mu\text{mol/kg BW/d}$ , which exceeds at least by a factor 3 the normal doses obtained from diet only. Isoflavone consumed as a supplement to achieve such doses would then have to be considered in future human clinical trials. Considering these data and conclusions altogether, isoflavones do have a positive effect in preventing bone loss and further replication of these data with relevance to humans could lead to consider isoflavones as bone-sparing compounds.

### c. Summary

As observed with cancer, isoflavones may offer osteoporosis protection. In this case, pre-, peri- or postmenopausal women as well as the dose of isoflavone, baseline bone mineral density and bone mineral content, and time of treatment should be carefully selected to investigate solely the role of isoflavones in preventing this chronic disease.

## 5. Isoflavones and menstrual cycle and menopause

Isoflavones have received much attention related to estrogen-dependent diseases and their mechanisms, because of the structural similarities that these compounds have with estrogen (Figure 3). Thus, isoflavones and some of their metabolites have been studied for binding to estrogen receptors (Setchell and Adlercreutz, 1988).

Isoflavones have among many other mechanisms, a weak estrogenic mode of action, and they can act as an agonist or antagonist compared to estrogens, depending on the tissue or organ reached and receptor bound (Jordon, 1990; Brann et al. 1995). Song et al. (1999) compared the estrogenic activity and estrogen receptor affinity of daidzein, genistein and glycitein compare to 17 $\beta$ -estradiol (the main estrogen hormone in premenopausal women) and diethylstilbestrol DES (synthetic estrogen). Using a mouse uterine enlargement assay

and estrogen receptor relative affinity, they found that  $17\beta$ -estradiol and DES were the most potent compounds and that isoflavones act as weak estrogens. They showed a relative affinity for the estrogen receptor that is 200, 3700 and 3600 times lower than  $17\beta$ -estradiol for genistein, daidzein and glycitein, respectively. In addition, daidzein estrogenic relative potency was known to be lower than that of genistein by a factor of 5 (Farmakalidis and Murphy. 1985), but the relative potency of glycitein was found to be 3 times higher than genistein. Although glycitein is found as in minor proportions in soyfoods, this stronger estrogenic activity deserve further attention (Song et al. 1999).

Estrogens and estrogen-like compounds bind to two families of estrogen receptors, ER  $\alpha$  and  $\beta$ , the second being recently discovered as being a receptor bound mostly by isoflavones and non-steroidal estrogens (Kuiper et al. 1997&1998). ER $\alpha$  is found in great proportions throughout the human body, especially in the adrenal gland, kidney, breast, uterus, ovaries and testes. The presence of ER $\beta$  has been shown in the brain, thymus, lung, bladder, bone, breast, uterus, ovaries and prostate (Setchell and Cassidy. 1999). The discovery of this receptor, along with the dual action of isoflavones in organs possessing either one or both receptors led to endless possibilities about the metabolic and health-related mechanisms of action of isoflavone.

Two of the main processes in women depending on estrogen metabolism that have been investigated with regard to isoflavones are menstrual cycle and menopause.

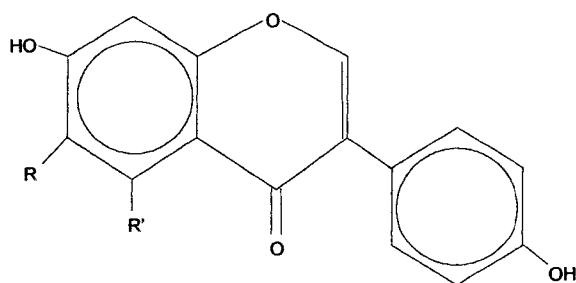


Figure 3: Chemical structure of isoflavone.

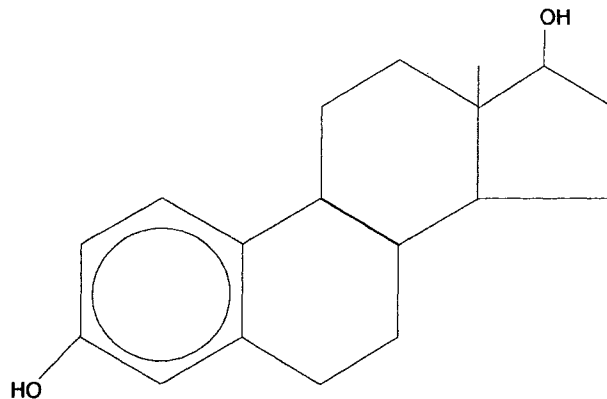


Figure 3 (continued): Chemical structure of estradiol

a. Menstrual cycle

Cassidy et al. (1994) examined the effect of 1 month supplementation of soy protein (60 g protein or 45 mg isoflavone/d;  $2.4 \mu\text{mol/kg BW/d}$ ) compared to a control diet without isoflavone on the menstrual cycle of 6 premenopausal women (mean age 24 years). Significantly delayed menstruation (1 to 5 d in 5 subjects) and increased follicular phase ( $2.5 \pm 1.6\text{d}$ ) but not luteal phase was observed with isoflavone intake. LH and FSH levels were significantly lower ( $21.2 \pm 12.7$  vs.  $7.1 \pm 2.6$  U/L and  $14.6 \pm 5.6$  vs.  $7.8 \pm 4.6$  U/L for LH and FSH levels of control vs. soy diets, respectively). Plasma estradiol also increased significantly during the luteal phase of the soy treatment. These results showed that isoflavones act as an anti-estrogen with respect to menstrual cycle. Cassidy also mentioned epidemiological data showing that Asian women have longer menstrual cycles and lower rates of breast cancer compare to Caucasian women (Treolar et al. 1970), suggesting a possible connection between hormonal modulation induced by isoflavones and prevention of breast cancer.

Lu et al. (2000) also examined hormonal levels of premenopausal women (n=10; mean 30 years of age) during a month supplementation of soy milk providing an average of 150 mg total isoflavone/day (7.9  $\mu\text{mol/kg BW/d}$ ). They found a significant decrease in 17 $\beta$ -estradiol (25%) and progesterone (45%) compare to baseline measurements previous to the study. No changes in LH, FSH or menstrual cycle length were found, although the increase in follicular phase was marginally significant ( $p=0.06$ ). As observed in Cassidy et al. (1994), the range of urinary excretion was very large and varied from 9.1 to 37.6% of ingested dose. Secondary analysis showed that decrease in 17 $\beta$ -estradiol was proportional to urinary excretion of isoflavones, which in turn was influenced by age.

Considering that there is a difference in mean age between the studies of Cassidy et al. (1994) and Lu et al. (2000) and that urinary isoflavones and possibly hormonal levels may be influenced by age, this could explain why some significant results found in Cassidy's study were not reproduced in Lu's study. High levels of circulating 17 $\beta$ -estradiol have been associated with increased risks of breast cancer (Hankinson et al. 1995), suggesting that isoflavones may protect against breast cancer by modulating sexual hormone profiles.

Isoflavone intake and its consequences in hormonal levels and menstrual cycle length tested over several menstrual cycles also need further investigation. Duncan et al. (1999) examined the effect of no (control: 0.15 mg or 0.5  $\mu\text{mol/kg BW/d}$ ), low (1 mg or 3.7  $\mu\text{mol/kg BW/d}$ ) or high (2 mg or 7.4  $\mu\text{mol/kg BW/d}$ ) isoflavone intake (from soy protein powder) in 10 pre-menopausal women. In a randomized cross-over design, each diet was consumed for 3 menstrual cycles. Compared to control, the low isoflavone diet significantly decreased plasma LH ( $26.6 \pm 1.8$  vs.  $19.1 \pm 2.0$  IU/L;  $p=0.009$ ) and FSH ( $4.62 \pm 0.2$  vs.  $3.92 \pm 0.2$  IU/L;  $p=0.04$ ). The high isoflavone diet significantly reduced DHEA-S ( $4566 \pm 521$  vs.  $4408 \pm 476$  nmol/L;  $p=0.02$ ) and free  $T_3$  ( $3.46 \pm 0.1$  vs.  $3.27 \pm 0.11$  pmol/L;  $p=0.02$ ). Moreover, isoflavone consumption did not influence endometrial thickness as assessed by histology (no detailed data provided). The authors concluded that isoflavone may not be the primary factor responsible for altering the hormonal profile and decreasing the risk of cancer in premenopausal women. In another study from the same group (Xu et al. 2000a), the hypothesis that isoflavones may decrease estrogen metabolites (16 $\alpha$ -(OH) estrone, 4-(OH) estrone and 4-(OH) estradiol) possibly related to an increase in cancer risk was investigated.

Using the same study design and diets as Duncan et al. (1999), 18 premenopausal women were assessed for urinary metabolites of estrogen. They used gas chromatography-mass spectrometry to analyze urine collected for 72 h at baseline and during the last 3 days of each feeding period. Compared to control, the low isoflavone diet ( $3.7 \mu\text{mol/kg BW/d}$ ) significantly increased the  $1/16\alpha\text{-(OH)}$  estrone ratio ( $3.82 \pm 0.33$  vs.  $5.09 \pm 0.38$ ) while consumption of  $3.7$  or  $7.4 \mu\text{mol/kg BW/d}$  decreased urinary  $4\text{-(OH)}$  estrone ( $1.93 \pm 0.10$ ;  $1.63 \pm 0.10$ ;  $1.51 \pm 0.10$  nmol/24h urinary excretion for control, low and high isoflavone diet, respectively). Compared with baseline values, all three soy protein diets significantly increased the ratio of urinary  $2/4\text{-(OH)}$  estrone ratio ( $3.80 \pm 0.11$ ;  $4.67 \pm 0.24$ ;  $5.14 \pm 0.25$  and  $5.05 \pm 0.24$  for baseline, control, low and high isoflavone diets, respectively) and significantly decreased genotoxic/total estrogens ratio ( $0.16 \pm 0.01$ ;  $0.14 \pm 0.01$ ;  $0.12 \pm 0.01$  and  $0.13 \pm 0.01$  for baseline, control, low and high isoflavone diets, respectively). The authors concluded that isoflavones along with other components found in soy protein exerted a cancer-protective effect by decreasing the amount of genotoxic estrogen metabolites. However, isoflavones per se may not have been the major components responsible for these effects.

When studied over a short period of time (1 month), it seemed that isoflavones exerted a significant effect in modulating hormonal profile towards a cancer-protective effect. However, more elaborate studies done over several menstrual cycles did not give significant results about the role of isoflavones in improving premenopausal estrogenic profile. Thus, the actual role of isoflavones in modulating hormonal profile is still controversial and deserves further investigation.

#### b. Menopause

The effects of isoflavone in postmenopausal women have showed modestly promising data, suggesting that isoflavone may be a potential candidate as an alternative to hormone therapy (HT, including estrogen and progesterone different from Estrogen therapy or ET that contains only estrogens), which has been associated with a 50% decrease in coronary heart disease risk (Stampfer and Colditz, 1991), but also with an increase in risk of breast cancer. Possible associations between soy intake and decreased risk of cancer,

atherosclerosis or osteoporosis are described in their respective sections. In addition, other menopausal-related symptoms have been studied with respect to soy and isoflavone intake. Using a double-blind, randomized, cross-over study (12-weeks long), Dalais et al. (1998) compared the effect of soy, linseed and wheat-based diets on several factors associated with menopause. Compared to baseline measurements, hot flushes were reduced by 22%, 41% and 51% for soy, linseed and wheat, with the last two being statistically significant. Only soy produced a significant increase in vaginal cytology maturation index (~103%, based on graphical data) and bone mineral density (5.2%). Reduction of hot flushes by wheat was not expected. In addition, the authors found 10 to 30-fold difference in urinary isoflavone excretion, but did not analyze the data based on apparent absorption, which could have resulted in different conclusions. The lack of further analysis based on urinary excretion limited the interpretation of the data.

More recently, Crisafulli et al. (2004) examined the effect of 1 year of dietary genistein supplementation (45 mg/d; 2.4  $\mu\text{mol/kg BW/d}$ ; other bioactive compounds not mentioned) on menopausal syndromes compare to estrogen progesterone therapy and placebo (n=30/group). Compared to a placebo, they found a decrease of hot flushes by 22 and 53% after 3 months, 29 and 56% after 6 months and 24 and 54% after 12 months for genistein and estrogen therapy treatments (overall  $p < 0.01$  for both treatment groups at all time points), respectively. In addition, endometrial thickness was similar in all 3 groups after 12 months. This study showed the positive impact of genistein on menopausal symptoms, without a negative impact on endometrial thickness.

In 2000, Pino et al. looked at the relationship between isoflavone supplementation and sex hormone binding globulin (SHBG) levels, also known to decrease with menopause. Ten postmenopausal women were provided 69mg isoflavones/d (3.65  $\mu\text{mol/kg BW/d}$ ) for 10 weeks, while 10 others were given a placebo. SHBG levels were compared to circulating plasma isoflavone concentrations, which is a better way to assess outcomes related to these compounds where bioavailability is widely different from one subject to another. They found a direct correlation between plasma isoflavones and percent increase in SHBG ( $r=0.84$ ;  $p < 0.001$ ). In addition, all women with circulating isoflavone concentration above 0.6  $\mu\text{mol/L}$  had at least a 30% increase in SHBG level. However, not all studies have shown a positive

impact of isoflavone on menopausal symptoms. Penotti et al. (2003) fed 10 postmenopausal women for 6 months a daily dose of 72 mg isoflavone or 3.8  $\mu\text{mol/kg BW/d}$  (controls,  $n=10$ , were fed placebo) and found no significant difference in hot flush reduction, endometrial thickness or pulsatility index of uterine arteries.

Duration of the studies, mean age of the subjects, isoflavone sources and doses may have contributed to the disparate conclusions of the studies described above. Statistical power may also be of importance. Crisafulli et al. (2004) found significant results with 30 subjects while Penotti et al. (2003) found no effect with 10 subjects. Nevertheless, isoflavones seem overall to exert a positive health effect on post- or peri-menopausal women and no signs of toxicity have been reported. Hence, isoflavones may indeed be a good candidate as an alternative to HT, but more research is needed.

## 6. Isoflavones and infant health

Short- and long-term health effects as well as toxicity of isoflavone intake by infants and children have also been the focus of several studies. Murphy et al. (1997) analyzed isoflavone concentration in several soy-based formulas and found a range of 214 to 267  $\mu\text{g/g}$  dry weight of isoflavones expressed as aglycone, which correspond to a daily intake of 5 to 12  $\text{mg/kg body weight}$  (18 to 44  $\mu\text{mol/kg BW/d}$ ) depending on the volume consumed by infants. The use of soy-based infant formula started more than 100 years ago to children with immunoglobulin E-mediated cow's milk allergy, lactose intolerance, galactosemia and as a vegetarian human milk substitute (Merritt and Jenks. 2004).

Prenatal exposure of isoflavone has been assessed in one study done by Adlercreutz et al. (1999). Asian pregnant women consuming a traditional diet (mean plasma isoflavone concentration was 233  $\text{nmol/L}$ ) showed that fetuses were exposed to significant concentrations of isoflavone as reflected in the cord blood (299  $\text{nmol/L}$ ) and amniotic fluid (223  $\text{nmol/L}$ ), which demonstrated that isoflavone can pass the placental barrier.

Setchell et al. (1997) found that in 4-month-old infants consuming soy-based formula, the mean daily intake of total isoflavone was 16 to 29  $\mu\text{mol/kg BW}$ . On the other hand, isoflavone concentration in breast milk of women consuming soy foods was  $5.6 \pm 4.4 \mu\text{g/L}$ ,

which caused minimal exposure of infants to isoflavones ( $<0.01 \mu\text{mol/kg BW/d}$ ) (Setchell et al. 1998).

Isoflavone intake from soy-based formula in infants per kg body weight was higher than in adults consuming soy products (30 g soy protein/d) by a factor 6 to 11 (Setchell et al. 1997; Franke et al. 1998) as reflected in plasma samples of soy formula fed infants (mean 980  $\mu\text{g/L}$ ; range 552 to 1775  $\mu\text{g/L}$ ). The greater apparent absorption has been hypothesized to be related to an immature gut microflora (lower bacterial degradation), a lower renal clearance (longer plasma half-life), but also partly to a more continuous feeding of greater proportion of isoflavones coming from soy formula solely (Setchell et al. 1998).

Because of these bioavailability data and the fact that isoflavones have potent estrogenic or anti-estrogenic activity, special attention has been brought to toxic or greater health-promoting effects of isoflavones in infants with respect to growth, reproductive, neurobehavioral and immune development as well as thyroid metabolism. However, no epidemiological studies have shown that pre- and neo- natal exposure to isoflavones resulted in toxicity signs. Asian women consuming high levels of isoflavone have been reported to carry normal pregnancy and delivery, while babies fed soy formula have not shown any clinical signs of disease or toxicity that could be associated with isoflavone intake (Badger et al. 2002). A follow-up study performed by Strom et al. (2001) showed that adults ( $\sim 20$ -30 y;  $n=811$ ) that were fed either soy ( $n=248$ ) or cow's milk ( $n=563$ ) as infants (0 to 4mths of age) did not show any significant difference in pregnancy outcomes, height, weight, age of sexual maturation, menstrual cycle length or missed period. Reproductive and non-reproductive parameters were also similar between the two groups. Neurobehavioral parameters, expressed by educational outcome (highest degree obtained) were not significantly different between the two groups. This showed that soy intake at a young age did not affect growth and development, and did not carry any long-term effect in young humans. The downside to that study was that subjects were too young (20-30 years of age) to develop chronic diseases so that the effects of isoflavone on cancer, diabetes, menopausal symptoms could not be evaluated, but deserve to be investigated.

In animals, Badger et al. (2001) found that rats injected with genistein (1850  $\mu\text{mol/kg BW/d}$ ) at days 2,4 and 6 after birth developed significantly less mammary tumors induced by



DMBA than controls over 110 d (30% decrease). If extrapolated to humans, this study showed that early exposure to isoflavones may indeed protect against chronic disease later in life. Actual human studies need to be done to confirm these data.

## 7. Isoflavone toxicity

Very few studies have concluded that isoflavone may show signs of toxicity. In animals, Hartley et al. (2003) fed isoflavones (150 mg/kg diet; 55  $\mu\text{mol/kg BW/d}$ ) to male Wistar rats ( $n=16$ ) for 18 d (14d as a group + 4 d in individual housing). Controls followed the same protocol and fed a diet without isoflavone. Animals fed isoflavones spent significantly less time in active social interaction measured as a social interaction test based on time spent grooming, sniffing, following, boxing and wrestling. In addition isoflavone intake was correlated with significantly higher stress-induced plasma corticosterone (100 vs. 30 mg/mL) and stress-induced plasma vasopressin concentration ( $\sim 6$  vs. 1 pmol/L). Stress was induced using an elevated plus-maze apparatus with 2 open and 2 closed arms. Therefore, it seemed that isoflavone had a negative behavioral effect on animals.

Yellayi et al. (2002) showed that isoflavones may have a negative impact on thymus. Subcutaneous injection of genistein (2, 8, 20, 80, 200 mg/kg body weight or 7.5, 29, 74, 296 and 740  $\mu\text{mol/kg BW/d}$ ) in ovariectomized C57BL/6 mice decreased thymus weight compared to control in a dose response manner with the 4 highest doses being significantly different from the control (17 to 78% decrease) which had a subsequent negative effect on immune function, especially humoral immunity. Only 7.5 and 29  $\mu\text{mol genistein/kg BW/d}$  doses have a relevance to humans in terms of dietary doses. Thus, since most of the toxic effects were observed with 74 and 296  $\mu\text{mol/kg BW/d}$ , doses that were injected subcutaneously, the relevance of these data to human may not be of significance.

McMichael-Phillips et al. (1998) looked at 48 women with benign or malignant breast disease. Subjects were divided in two groups, one control and one fed 60 g ground texture vegetable protein containing 45 mg isoflavone for 14 d. Soy-fed subjects had a significant increase in proliferation of breast tissue ( $0.91 \pm 0.9$  vs.  $1.74 \pm 1.72$  labeling index for control vs. soy, respectively), which is associated with increased risk of breast cancer. Progesterone

receptors were also higher in the soy vs. control group ( $19.32 \pm 9.04$  vs.  $14.85 \pm 8.62$  labeling index). This agonist effect of isoflavone is in agreement with Pollar et al. (1997) in a study in rats focusing on prostate cancer. Isoflavones were cancer-protective before cancer occurs while they were cancer-enhancing when cancer is already present. Indeed, isoflavone (33.8mg total isoflavone/kg diet) fed after cancer initiation did not produce a significant protective effect compare to the same treatment applied before cancer initiation.

In addition, Hargreaves et al. (1999) studied 84 premenopausal women fed control or soy (45 mg isoflavone/d) for 14 d. Even though breast cell proliferation was not increased with soy, pS2 (marker of estrogenic response) levels significantly increased while nipple aspirate levels of apolipoprotein D decreased in the treatment group confirming the weak agonist estrogenic activity of isoflavone on cancer once it has already developed.

Another study done by Bloedon et al. (2002) focused on postmenopausal women (n=24). Four single doses of isoflavone based on genistein content (2, 4, 8 and 16 mg/kg body weight or 7.4, 14, 29 and 59  $\mu\text{mol/kg BW/d}$ ) were used in two preparations, both containing mostly isoflavones in their aglycone form. Isolated episodes of edema, nausea and breast tenderness have been hypothesized by the authors to be linked to isoflavone intake, mostly because these events occurred a short period of time after ingestion of treatment diets. From one of the isoflavone preparations, subjects experienced (regardless of the dose ingested) an average of 7% increase in systolic and diastolic pressure along with a 32% average decrease in neutrophil counts 24 h after isoflavone ingestion. The conclusions of possible toxicity may be irrelevant considering that isoflavone were fed at pharmacological and not dietary doses (a high isoflavone intake associated with a typical Japanese diet may not exceed 1 mg/kg body weight/d (Munro et al. 2003)). The doses fed here may be achievable through supplement intake, but the studies on health-related effects described in this review were based on isoflavone intake coming from the diet and not supplements. Studying health-effects of high doses of isoflavones related to individuals consuming supplements may lead to a whole new set of studies, hypothesis and rules that could lead to totally different conclusions. This has not been investigated yet, but certainly would deserve attention, considering the growing popularity of these compounds.

### C. Isoflavone metabolism and bioavailability

#### 1. Overview of isoflavone metabolism

The actual process of isoflavone absorption is still somewhat obscure. The structure of isoflavone aglycones (MW=254, 270 and 284 g/mol for daidzein, genistein and glycitein, respectively) is neither hydrophilic nor lipophilic and there is no evidence that they are absorbed by facilitated or active transport. Due to their low molecular weight, isoflavones could then be absorbed through diffusion, but this remains to be verified (Birt et al. 2001).

To investigate cellular uptake and absorption of isoflavone aglucons and glucosides, Murota et al. (2002) used Caco-2 cell monolayers as a model of human intestinal epithelium. When incubated with 10  $\mu\text{mol/L}$  of either daidzein or genistein on the apical (i.e., mucosal side), 20% and 15% of the dose applied for genistein and daidzein, respectively were found on the baso-lateral side after 2 h of incubation and the presence of glucuronides and sulfate conjugates indicated that isoflavones may be conjugated right after absorption and not exclusively in the liver. The same incubation performed with daidzin and genistin revealed that only daidzein and genistein as well as their conjugates were found at the baso-lateral side, supporting the idea that isoflavone glucosides cannot be absorbed intact. Moreover, only 1.5% of the applied dose was found on the basolateral side, meaning that glucosides absorption may be slower than aglycones (data based on 0-2 h only after isoflavone injection). The overall percentage of the applied dose found on the basolateral side was not included in the study which focused only on 0-2 h incubation.

In vivo, Setchell et al. (2002) failed to find any isoflavone glycoside at 1, 2 or 8 h in plasma samples collected from 12 women ingesting 50 mg of either daidzin or genistin. Due to a fast appearance of isoflavones in plasma after ingestion (maximum plasma peak reached at 6 h or sooner in humans; Setchell et al. 2002; Xu et al. 1994), it was hypothesized that isoflavone glycosides were cleaved by human intestinal enzymes and absorbed in the small intestine. Indeed, Day et al. (2000) found that lactase phlorizin hydrolase, a membrane-bound, family 1  $\beta$ -glycosidase found on the brush border membrane of mammalian small intestine, was able to cleave the sugar moiety of daidzin and genistin with a greater  $V_{\text{max}}$  for

genistin (~2.8 U/mg LPH) than daidzin (~0.9 U/mg LPH). This cleavage process apparently delayed the absorption compared to ingested isoflavone aglycones. Setchell et al. (2001) found in humans a peak plasma concentration of 5.2 and 6.6 h for genistein and daidzein, but it took 9.3 and 9.0 h for genistin and daidzin to appear in the plasma as their respective aglycone and conjugates forms. Plasma area under the curve was similar between glucosides and aglycones. Zheng et al. (2004) also found no significant difference in urinary excretion of women fed soygerm (~94% glucosides;  $50.8 \pm 5.6\%$  urinary total isoflavone as percentage of ingested dose) or fermented soygerm flour (~88% aglycones;  $51.5 \pm 6.1\%$  urinary total isoflavone as percentage of ingested dose). These in vivo data are in agreement with the data observed in vitro by Murota et al. (2002).

In animals, Andlauer et al. (2000a) used an *ex-vivo* rat small intestine model. Doses of 5.9, 12.0 and 23.8  $\mu\text{mol}$  genistin were applied to the luminal side. About 15% injected dose was found on the basolateral side, mostly as glucuronide (11.6%) with small amounts of genistein (1.9%) and genistin (1.3%). The latter finding was surprising, but considering this *ex-vivo* model, it was possible that a leak in the system could have resulted in the passage of intact genistin to the basolateral side. Despite this ambiguous situation, the authors concluded that cleavage of the glucosidic form seemed required to facilitate a greater absorption and that conjugation of isoflavone was a process also occurring at the intestinal level.

Distribution of isoflavones in the different tissues and organs of the body occurs rapidly, but storage in organs or tissue is virtually nonexistent. In an in vivo system, Coldham and Sauer (2000) used [ $^{14}\text{C}$ ]-genistein fed at a dose 4 mg/kg body weight (14  $\mu\text{mol/kg}$  BW) to 5 male and 5 female Wistar rats. At 24 h after ingestion of the genistein dose, no significant radioactivity was detected in heart, lung, brain, testes or ovaries, bone, fat or thymus.

After absorption, isoflavones undergo endogenous biotransformation by UDP-glucuronosyltransferase (UGTs) and sulfotransferases in the intestinal mucosa, liver and other organs (Barnes et al. 1998), which modifies its chemical structure and solubility, thereby influencing its distribution, storage and excretion (Birt et al. 2004). Glucuronide and sulfate conjugates made up about 70-80% and 20% in urine, respectively and 50-60% and 20-30% in plasma, respectively in women fed soymilk (n=6) (Zhang, 2000). In addition,

greater amounts of isoflavone aglycone were recovered from 0-24 h plasma samples (~20-25% ingested dose) compared to 0-24 h urinary excretion (<10% ingested dose). The fact that the amounts of total isoflavones recovered from urine vs. plasma as percentage of the ingested dose somehow do not match may be due to enterohepatic circulation (Sfakianos et al. 1997), where more than half of the ingested isoflavone was excreted in the bile after conjugation. Limited evidence and further investigation are crucial to confirm this hypothesis. Sfakianos et al. (1997) used anesthetized female rats with biliary cannulas to measure isoflavones biliary excretion. Genistein infused into the duodenum was recovered as 7-O- $\beta$ -genistein glucuronide and 75% of the infused dose was recovered within 4h. Moreover infusion of genistein glucuronide in the duodenum resulted in a slower biliary excretion of the same compound, indicating that like glucosides, isoflavones endogenously conjugated have to be cleaved prior to reabsorption, either by intestinal or microbial enzymes.

Back in the intestine, isoflavones are deconjugated by gut microbial glucuronidase and sulfatase to release the aglycone form, part of it being possibly reabsorbed through enterohepatic circulation (Sfakianos et al. 1997). Isoflavones not undergoing this process are submitted to microbial degradation and metabolite formation, some of which can also be absorbed to exert weak estrogenic or anti-estrogenic effects, while the rest undergoes fecal excretion (See figure 4; from Hendrich and Murphy, 2001).

From the data described above, humans and animals share great similarities in absorption, biotransformation and excretion of isoflavones. Plasma kinetics and urinary distribution of isoflavones (bioavailability) as well as microbial metabolism of these compounds are discussed in the next section.

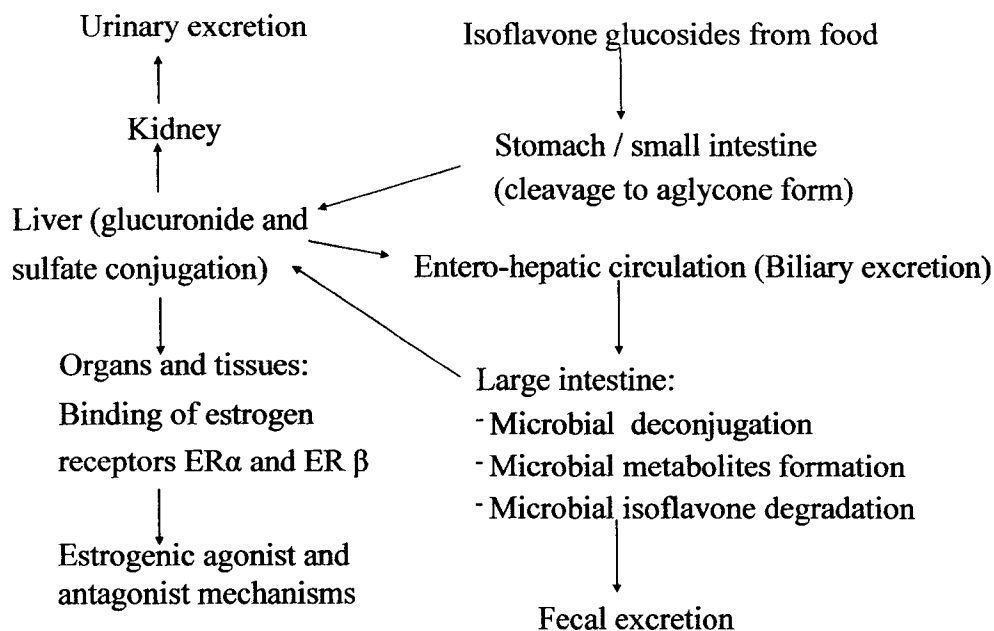


Figure 4: Overview of metabolism (adapted from Hendrich and Murphy. 2001)

## 2. Overview of isoflavone bioavailability

Bioavailability is defined as the proportion of a compound that appears in plasma over time when the compound is administered orally. It is also defined nutritionally as the proportion excreted in the urine and feces compared to the amount ingested (Birt et al. 2001). Depending on the chemistry of the molecule (fat- or water-soluble), bioavailability may also be affected by storage, especially in fat, when the compound is lipid-soluble. Considering that isoflavones are conjugated after absorption, they become more water soluble and storage of these compounds may then be very little or none (Birt et al. 2004). Consequently, bioavailability of isoflavones focuses mostly on plasma and urinary kinetics as well as microbial metabolism. Food matrices in which isoflavones are found and chemical differences that separate daidzein, genistein and glycitein (Figure 1) have also been studied with respect to possible variations in isoflavone bioavailability (Birt et al. 1994; Xu et al 2002).

### a. Animal models

To study bioavailability of isoflavones with possible extrapolation to humans, a valid animal model needs to be established. So far, rats have been one of the most investigated animal models to study kinetics and fate of isoflavones. Using isolated rat small intestine as an *ex vivo* animal model, Andlauer et al. (2000b), studied the absorption rate and biotransformation of isoflavones daidzin and genistin derived from pre-digested tofu (1184 nmol genistein expressed as aglycone/g tofu and 572 nmol daidzein expressed as aglycone/g tofu). Tofu also contained small amounts of malonyl-isoflavone and isoflavone aglycone. Of these compounds, 8% genistein and 8.9% daidzein appeared at the vascular side, either as aglycone, glucuronide and glucoside. A 3- and 2-fold increase in the aglycone genistein and daidzein, respectively was found at the luminal side. However, no sulfate conjugate were found in this experiment. In another experiment with only genistin applied in an isolated rat small intestine, Andlauer et al. (2000b) found an absorption rate of about 15%, with glucuronides>aglucons>glucosides found on the vascular side. These two studies showed that daidzein and genistein absorption rate are similar and that biotransformation occurs during the absorption process. However, the absence of sulfate conjugate indicated that rat may not be able to produce this conjugate, or this process is restricted exclusively to the liver. The presence of isoflavone glucoside on the vascular side was surprising and lead to the conclusion that rat may be capable of absorbing isoflavone glucoside without cleavage. On the other hand, the very small proportion of isoflavone glucosides found may have been related to a leakage in this *ex vivo* system.

In an *in vivo* system, Coldham and Sauer (2000) used [ $^{14}\text{C}$ ]-genistein fed at a dose 4 mg or 14  $\mu\text{mol/kg}$  body weight in 5 males and 5 females Wistar rats. Mass balance of radioactivity showed that 67 and 66% [ $^{14}\text{C}$ ] were recovered in the urine and 30 and 36% in the feces for males and females, respectively. In the first 2.5 h, male rats had significantly higher isoflavone concentration and showed a higher secondary plasma peak at 6-8 h after ingestion compared to females. On the other hand, females showed a higher liver [ $^{14}\text{C}$ ] at 2h and 7 h compare to males; this could explain the lower plasma levels found in females. Mass

spectrophotometry analysis showed that glucuronides and sulfate conjugates were present in both sexes. No glucosides were found in plasma so some of the *ex vivo* data obtained by Andlauer et al. (2000b) were not reproducible *in vivo*. This study is of importance, because it showed several similarities between rats and humans bioavailability of isoflavones (absorption process, conjugation and plasma kinetics and gender difference). However, only mass balance was measured in urine and feces so that radioactivity from metabolites produced by gut microorganisms was not separated from the radioactivity of “intact” isoflavones aglycones and conjugates.

King (1998) compared the bioavailability of daidzein and genistein in male Wistar rats fed a soy extract providing 74  $\mu\text{mol}$  genistein and 77  $\mu\text{mol}$  daidzein/kg BW. 48 h urinary excretion of daidzein and genistein were  $17.4 \pm 1.2\%$  and  $11.9 \pm 1.1\%$  of the ingested dose, respectively, while fecal excretion was  $2.3 \pm 0.5\%$  and  $3.4 \pm 0.4\%$ , respectively. As observed in humans (Xu et al. 1994 & 1995; Setchell et al. 2003), daidzein is more bioavailable than genistein and the range of urinary excretion is comparable to human data. Therefore, not only are rats an interesting model to study bioavailability, but also the gut microbial metabolism of humans and rats may share some similarities in degrading or metabolizing isoflavones.

With respect to humans, King et al. (1996) also compared the bioavailability of genistein glucoside vs. aglycone (20 mg or 74  $\mu\text{mol}$ /kg BW fed to male Wistar rats). Genistein was absorbed faster than genistin as reflected in plasma concentration ( $11.0 \pm 2.3 \mu\text{mol/L}$  vs.  $4.93 \pm 0.22 \mu\text{mol/L}$  of genistein 2 h after genistein or genistin ingestion, respectively). As a consequence, genistein-fed animals had a faster urinary excretion. On the other hand, 48 h recovery from plasma, urine or feces was similar between the two groups, indicating that bioavailability of isoflavone aglycones and glucosides are equivalent. The same phenomenon has also been observed in humans (Setchell et al. 2001). So many similarities in data between rat and human data could lead to the establishment of rats, or rodents in general, as valid models to study isoflavone bioavailability.



## b. Humans

Many human studies have focused on understanding how isoflavones are metabolized in the body, with special focus on plasma kinetics as well as urinary and fecal excretion.

Xu et al. (1994) fed 12 women 3 doses of isoflavones (2.5, 4.8 and 7.4  $\mu\text{mol/kg}$  BW from soymilk powder; 44% genistein and 56% daidzein). Plasma levels of daidzein and genistein were similar to each other at time 6.5 and 24 h following ingestion at all 3 doses. Urinary recovery of daidzein and genistein were 21% and 9% of the ingested dose, respectively and fecal excretion was 1-2% ingested dose. The amounts of isoflavone recovered in the urine were proportional to the ingested dose (dose-response between amounts fed and those recovered in the urine). Total isoflavone excreted in the urine was higher in 0-12 h compared to 12-24 h following ingestion by a factor of 2 to 3, meaning that isoflavone absorption and metabolism is a fast process, occurring mostly in the first 12 h after ingestion and disappearing almost completely after 24 h. Based on urinary excretion, Xu et al. (1994) concluded that daidzein was more bioavailable than genistein. Setchell et al. (2003) also came to the same conclusion using [ $^{13}\text{C}$ ]-labeled isoflavones and found a urinary recovery of 30.1% and 9.0% of the ingested dose (0.4 mg of [ $^{13}\text{C}$ ] daidzein or genistein, n=16 premenopausal women) for daidzein and genistein, respectively.

Xu et al. (1995) performed a similar study to that of Xu et al. (1994) with three doses of isoflavones fed (3.4, 6.9 and 10.3  $\mu\text{mol}$  isoflavones/kg body weight; n=7 women), but looked at the individual results and found that 2 subjects had significantly higher fecal excretion of isoflavones compare to the 5 others (about 6% vs. 0.6% of the ingested dose, respectively and regardless of the dose fed). Forty eight hours urinary recovery was  $16 \pm 4\%$  and  $10 \pm 4\%$  of the ingested dose for daidzein and genistein, respectively in the 5 subjects with low fecal excretion. The two subjects with high fecal excretion had  $32 \pm 5\%$  and  $37 \pm 6\%$  urinary recovery expressed as a % ingested dose. As for plasma, subjects with high fecal and urinary excretion had plasma isoflavone level 2.5-fold higher compare to subjects who were low isoflavone excreters. This study established the principles of phenotypes of isoflavone bioavailability, in which people can be grouped as high apparent absorbers (high urinary, plasma and fecal isoflavone contents) or low apparent absorbers. Moreover, high

apparent absorbers do not seem to have as much gut microbial activity degrading isoflavones, because they are excreted intact and in greater level in the feces those subjects. The role of the gut microflora in determining the extent of isoflavone bioavailability became then a factor that could not be overlooked. These two studies performed by Xu et al. (1994 & 1995) did not consider bioavailability of glycitein, a minor, but still important soy isoflavones.

By feeding soymilk (4.5  $\mu\text{mol}$  total isoflavone/kg BW) to 14 subjects (7 males, 7 females), all in vitro fecal moderate degraders, Zhang et al. (1999b; erratum 2001) found that plasma genistein concentration was higher than that of daidzein, which in turn was higher than that of glycitein at both 6 and 24 h after ingestion by males ( $1.29 \pm 0.5$ ,  $1.78 \pm 0.83$  and  $0.22 \pm 0.08$   $\mu\text{Mol}$  at time 6h for daidzein, genistein and glycitein respectively) or females ( $1.04 \pm 0.61$ ,  $1.70 \pm 1.01$ ,  $0.20 \pm 0.08$   $\mu\text{Mol}$  at time 6 h for daidzein, genistein and glycitein respectively). However, urinary excretion of glycitein was the highest, followed by daidzein and genistein ( $48.6 \pm 23.1$ ,  $27.6 \pm 20.7$  and  $55.3 \pm 26.0$  % ingested dose over 48h after ingestion in males and females together for daidzein, genistein and glycitein, respectively). No significant difference was found in bioavailability data between males and females. This study showed that isoflavones were absorbed to a great extent, but urinary data supported the idea that genistein may be more susceptible microbial degradation compared to daidzein, so that less genistein is re-absorbed and found in the urine. In other words, overall absorption of daidzein is apparently greater than that of genistein. Lower glycitein plasma concentration was probably due to lower doses of that isoflavone fed, but high glycitein urinary excretion may be related to a low microbial degradation/high reabsorption phenomenon as hypothesized for daidzein. The presence of the methoxyl group on the glycitein molecule could be in part responsible for the data observed in this study, underlying the hypothesis that glycitein may be less susceptible to microbial degradation.

In another study, Lu and Anderson (1998) focused on sex and long-term (1 month) soymilk intake (200 mg total isoflavone/d or  $\sim 10$   $\mu\text{mol/kg}$  BW/d) with regard to urinary excretion of isoflavones and metabolites. They found that women ( $n=6$ ) excreted 24% and 66% of the ingested dose of genistein and daidzein respectively, while men ( $n=6$ ) excreted only 15% and 47%. After one month, women excreted less daidzein (14%) and genistein (45%) than at time 0 in the urine, while in men, isoflavone urinary excretion levels were

stable. With respect to equol production one man and one woman were capable of producing equol from daidzein at time 0. Moreover, 3 additional women but no additional men developed the capability to produce equol over the one month study. This study showed a sex difference in isoflavone bioavailability and the fact that in women, chronic exposure to soy may alter the gut microflora, leading to a higher degradation of isoflavones (thus less urinary excretion), and the production of new metabolites, such as equol. Men may have a more stable microflora, explaining the stability of urinary excretion and the absence of new subjects developing the capability to produce equol.

Separate studies conducted in men in women provided additional data to plasma kinetics of isoflavones. In women, Xu et al. (1994) found similar daidzein and genistein concentration in plasma 6.5 and 24 h after ingestion of soymilk. In men, Watanabe et al. (1998) observed a peak of genistein in plasma at 6h after ingestion of baked soybean powder, peak that was two-fold higher than the peak observed for daidzein. Plasma half-lives of daidzein and genistein were 7.75 h and 7.77 h in women (Setchell et al, 2003), and 8.36 h and 5.79 h in men (Watanabe et al. 1998), respectively. Unfortunately different isoflavone composition, subject numbers, handling of plasma samples and analysis differed from one study to the next, making comparison across studies impossible to make. Only Zhang et al. (1999b; erratum 2001) studied both sexes within the same study and found no gender difference in overall isoflavone bioavailability.

In summary, bioavailability of isoflavones is now fairly well understood, and data on plasma kinetics, urinary and fecal excretion are reproducible from one study to the other. Isoflavone absorption is a rapid process, in men or women, with a maximum plasma peak occurring within 6 h after ingestion. Only traces of isoflavone are detected in the urine after 48h, meaning that most of the isoflavones ingested are being excreted within 24-48 h. The data obtained from animal and human studies are also comparable, so that potential animal models could be validated to study isoflavone bioavailability and/or health related effects observed with these compounds in more detail. On the other hand, in both humans and animals, urinary plus fecal excretions are not equal to 100% of the ingested dose and most of the studies have found a cumulative urinary and fecal excretion not exceeding 60% (in many studies, even much less than that), suggesting that a great amount of isoflavones disappear in

this process. There is now good evidence that gut microorganisms are greatly involved in the process of bioavailability, as described in the following section.

### c. Isoflavones and gut microorganisms

Gut microorganisms play an important part in isoflavone bioavailability. Several aspects of the absorption/metabolism/degradation processes may be influenced by the presence of the gut microflora.

In order to be reabsorbed after biliary excretion, isoflavone conjugates must be cleaved. The same applies to any isoflavone glucosides that might have arrived intact in the ileum or large intestine. Bacteria play an important role in this cleavage process through  $\beta$ -glucosidase or  $\beta$ -glucuronidase activity. Previous *in vitro* work done by Hawksworth et al. (1971) showed that a variety of bacteria possess these enzymatic activities using *in vitro* incubation of groups of bacteria with various sugars, all differing in their glycosidic bonds from one another and measuring the different sugar breakdown over time (sucrose added for  $\beta$ -glucosidase activity, substrate of  $\beta$ -glucuronidase not provided).  $\beta$ -glucosidases have been found in Enterococci and to a lesser extent in Lactobacilli, Clostridia, *Bacteroides* and Bifidobacteria strains.  $\beta$ -glucuronidase activity is mostly due to Enterobacteria and to a much lesser extent by *Clostridium* and *Bacteroides*.

Xu et al. (1995), while studying bioavailability of isoflavones in 7 subjects, found that 2 individuals excreted significantly higher amounts of isoflavones in their urine and had significantly higher peak plasma concentration. This was the first indication that isoflavone bioavailability was not the same in all individuals. The role of the gut microflora in degrading more isoflavones in those who excreted less of it the urine have been hypothesized and confirmed by Hendrich et al. (1998). Men and women (n=20) were screened for *in vitro* fecal isoflavone degradation. After cluster analysis, they found three phenotypes of degradation for daidzein: high (n=5; k=0.299), moderate (n=10; k=0.055) and low (n=5; k=0.012). When rechecked after 10 months, the high (n=5; k=0.326) and moderate (n=4; k=0.073) phenotypes shown greater stability compare to low degradation phenotype (n=5; k=0.053). The same phenotypic profile was observed for genistein. High (n=5), moderate

(n=10) and low (n=5) degraders showed degradation rate constants of 0.299, 0.163 and 0.023, respectively. After 10 months, high (n=5), moderate (n=4) and low (n=5) degraders had mean degradation rates of 0.400, 0.233 and 0.049, respectively. Overall, 12/14 subjects who were re-examined after 10 months showed stability in their phenotyping. Subjects switching from one phenotype to another, along with greater  $k$  observed for each phenotype and isoflavone after 10 months were indicative of variation in the microflora, which probably would affect isoflavone bioavailability over time. In addition, genistein was overall degraded faster than daidzein, as reflected in mean rate constant values (0.023 vs. 0.163 for daidzein and genistein, respectively).

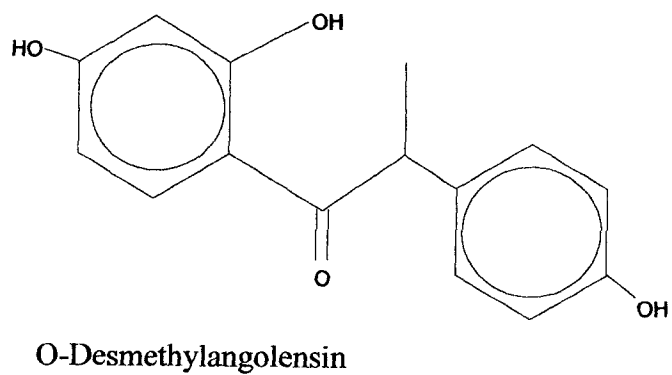
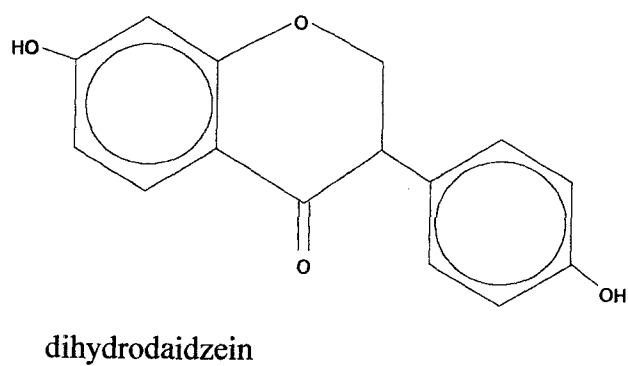
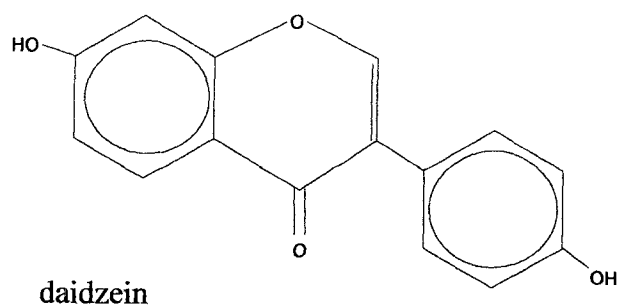
This difference observed *in vitro* could partly explain the difference observed in urinary excretion. Urinary excretion of daidzein is greater, apparently because of a lower degradation in the gut. Chemical structures of both isoflavones could also explain this difference: daidzein has 2 hydroxyl groups, while genistein has 3 at key positions (C-5,7,4) that makes it theoretically more susceptible to degradation. Griffiths and Smith (1972) compared microbial degradation of several phenolic compounds, including isoflavones, and found that the presence of a hydroxyl group in the 5 position was associated with greater microbial degradation. Genistein, but not daidzein, possesses a 5-OH group, which would then make it more susceptible to microbial degradation and in agreement with *in vivo* and *in vitro* data. As for glycitein, no published data on phenotypes of degradation or urinary excretion is available, but one would assume that this compound would be degraded or excreted among subjects according to phenotypes. Moreover, urinary excretion values of glycitein close to that of daidzein (Zhang et al. 1999b; erratum 2001) may lead to the hypothesis that both compounds would have similar patterns of gut microbial degradation. Finally, degradation phenotypes and bioavailability of glycitein in animals has not been elucidated yet.

Identification of isoflavone-degrading microorganisms has not been done yet. There have been a few studies focusing on single strains and their ability to degrade these compounds, but so far, no study has focused on the colonic/fecal microflora as a whole. Winter et al. (1989) showed that Clostridial strains were capable of cleaving the C-ring of the flavonoids quercetin, kaempferol and naringenin. Clostridial species may also be involved in

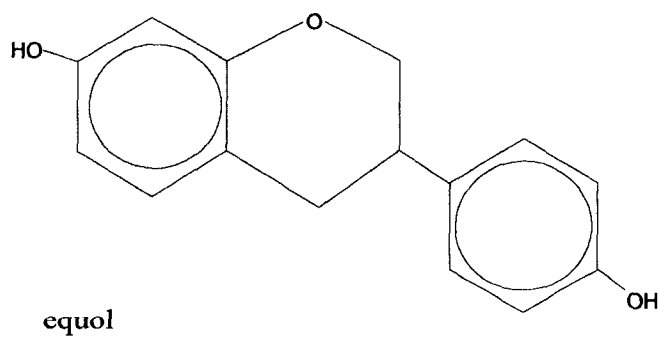
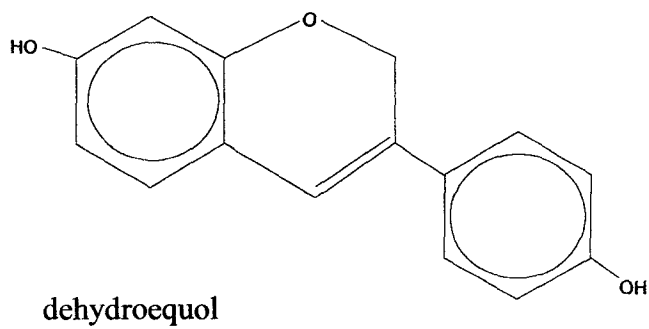
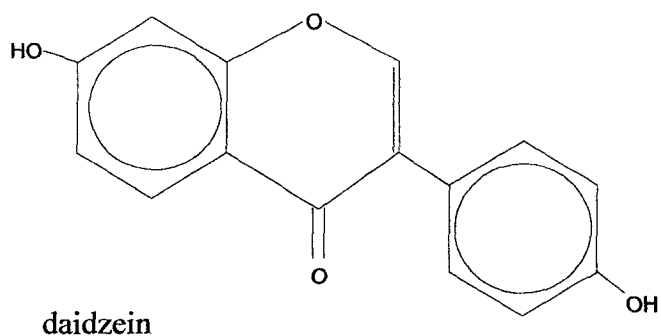
degrading daidzein. Hur et al. (2002) found that a *Clostridium sp.* isolated from human fecal samples (200 different unknown bacterial colonies cultured from fecal sample and each incubated with daidzein) was capable of transforming daidzein (400  $\mu$ M) into ODMA (112  $\mu$ M) over a 3d incubation. *Eubacterium limosum*, a strict anaerobe from the human intestinal tract, has been showed to transform glycitein (100  $\mu$ M) to 6,7,4'-trihydroxyisoflavone (Hur and Rafii. 2000). However, the amount of metabolite produced and the time to produce it were not mentioned, leading to a difficult extrapolation to humans. Schoefer et al. (2002) found that a pure strain of *Eubacterium ramulus* degraded 0.5 mM genistein completely in 4h into 6'-hydroxy-ODMA and 2-(4-hydroxyphenyl)-propionic acid. Daidzein was degraded over 12 h by *E. ramulus* to yield ODMA at an amount equal to 70% of the daidzein initially present. The metabolites produced were in agreement with those observed *in vivo* (Joannou et al. 1995) so that *E. ramulus* may degrade isoflavones. However, bacterial competition and colonic environment were not considered, despite being of great importance when studying gut bacterial degradation of isoflavones.

#### d. Gut microbial metabolites of isoflavones

Gut microflora are also responsible for converting isoflavones to metabolites, but the bacteria responsible for metabolizing these compounds as well as biological effects associated with these metabolites remain to be identified. Daidzein catabolism has been proposed to yield the metabolite dihydrodaidzein, itself transformed into O-desmethylangolesin (O-DMA) or equol. As for genistein, its microbial metabolism has been associated with the formation of dihydrogenistein, itself catabolized to 6'-hydroxy-O-DMA (Joannou et al. 1995). Most of these metabolites have not been fully understood and/or investigated from a microbial or health-related standpoint. Chemical structures of daidzein, genistein and their respective metabolites can be found on the next pages.

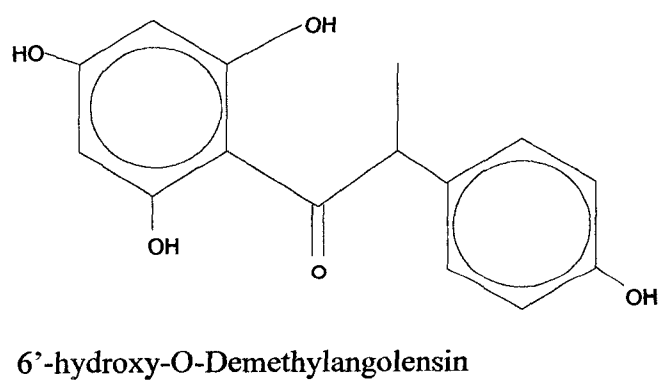
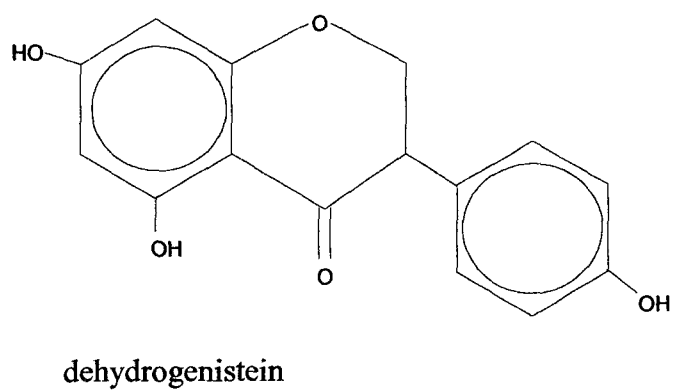
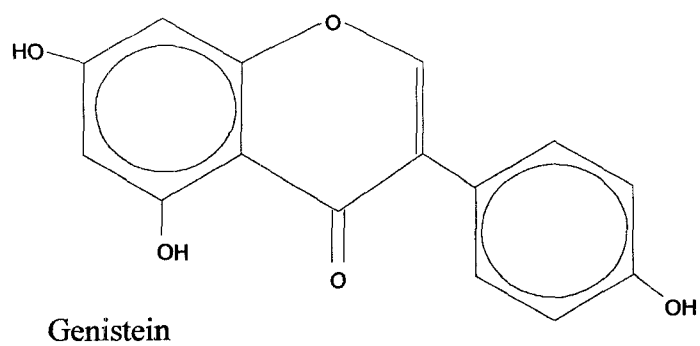


Chemical structures of daidzein, dehydrodaidzein and O-desmethylangolesin (O-DMA) according to the first possible pathway of daidzein microbial degradation



Chemical structures of daidzein, dehydroequol and equol according to the second possible pathway of daidzein microbial degradation





Chemical structures of genistein and its microbial metabolites: dihydrogenistein and 6'-hydroxy-ODMA.

One of these metabolites has brought some attention because of its weak estrogenic activity: equol, derived from daidzein. Lampe et al. (1998) showed that ~35% of subjects (n=60; 30 men and 30 women) are capable of producing equol after eating soybean for 3d and the range of equol production varied by a factor 10. Women who were equol producers had a significantly higher intake of energy from carbohydrate, plant protein, soluble and insoluble fibers. These correlations were not found in men. In addition, Lu et al. (1996) found that chronic soy exposure in women (n=6) resulted in 1 subject producing equol at baseline vs. 4 subjects one month later. Therefore, it is possible that chronic soy intake may modify the microflora or activate some bacterial enzymes responsible for equol production. The same study performed in men (Lu et al. 1995) showed that one subject was equol producer at baseline, but no additional subjects produced equol over a month chronic soy exposure. Therefore, equol production seems more susceptible to be activated in women than in men by chronic soy exposure. This sex-difference also suggests that the microflora of men may be more stable than that of women. Despite some attention brought to equol, its health or toxic effects still remain to be fully investigated. Selvaraj et al. (2004) studied the estrogenicity of equol in ovariectomized C57BL/6 mice (n=5/group; 30 d old) injected daily for 12 d with 0, 4, 8, 12 or 20 mg equol/kg BW/d (0, 16, 33, 50 and 83  $\mu\text{mol/kg BW/d}$ ) or fed 0, 500, 1000 ppm equol (0, 400, 800  $\mu\text{mol/kg BW/d}$ ) in AIN-93G diet. None of the dietary treatments had an effect on uterine weight while injected doses of 50 and 83  $\mu\text{mol/kg BW/d}$  increased significantly uterine weight by a factor 2 and 4, respectively compare to control. Dietary and injected equol both induced a dose dependent increase in endometrial thickness (~7  $\mu\text{m}$  in control up to 40  $\mu\text{m}$  with 83  $\mu\text{mol/kg BW/d}$  injected and 30  $\mu\text{m}$  with 800  $\mu\text{mol/kg BW/d}$ ; approximation from graphical data). The same phenomenon was observed with uterine epithelial proliferation (up to 90-fold and 20-fold increase with highest dose injected or fed compare to control, respectively). The authors concluded that equol was a weak estrogen, but extrapolation of injected doses to an in vivo human situation is impossible to make. On the other hand, the authors found concentrations of ~8  $\mu\text{M}$  plasma equol when fed orally at 800  $\mu\text{mol/kg BW/d}$  and compared it to normal human plasma levels of equol producers (only 1  $\mu\text{M}$ ). Therefore, the dose of equol applied was too high to extrapolate these results to humans with significance. Likewise, no soy products contain such doses of equol.

Only microbial degradation of daidzein is responsible for equol appearance in plasma and urine, which is one of the reasons why equol is such a difficult compound to work with to obtain significant extrapolable data. One of the few studies looking at equol and health related benefits in humans was done by Duncan et al. (2000). Premenopausal women who produced equol (n=5) were compared to non-equol producers (n=9) for plasma hormone and sex hormone binding globulin (SHBG) levels. They found that equol production was significantly associated with overall lower concentrations of testosterone ( $p<0.01$ ), dehydroepiandrosterone ( $p<0.01$ ), cortisol ( $p=0.03$ ), midluteal estrone ( $p=0.004$ ) and higher levels of SHBG ( $p=0.02$ ) and midluteal progesterone ( $p=0.002$ ). The authors concluded that equol producers had hormonal profiles consistent with reduced risk of breast cancer so that equol might directly (or indirectly) influence hormone production in a beneficial way.

In addition to the metabolites identified by Joannou et al. (1995), other by-products of isoflavone gut microbial metabolism have been identified. In Wistar rats, King (1998) found equol and 4-ethyl phenol to be the main metabolites from daidzein and genistein, respectively. However, equol was mostly produced 24-48 h after isoflavone ingestion while 4-ethyl phenol was produced between 0-24 h and reached a plateau between 24 and 48 h. This showed that metabolite production does not occur at the same time or at the same level, meaning that gut microorganisms metabolize each of the isoflavones differently. Using [ $^{14}\text{C}$ ] labeled genistein also in Wistar rats, Coldham and Sauer (2000) found that genistein can be metabolized into 4-hydroxyphenyl-2-propionic acid in males and dihydrogenistein in females. However, proportions of these metabolites as percentages of the ingested dose were not calculated in either study (King 1998; Coldham and Sauer. 2000) so that there are still some uncertainties as to how important these new identified metabolites actually are from metabolic and physiologic standpoints.

#### e. Factors influencing isoflavone bioavailability

Bioavailability has been extensively studied in both animals and humans, as described above. However, there may be factors that influence the extent to which isoflavones are

apparently absorbed or degraded. Diet, food matrix, food processing or gut transit time may be involved in modulating this process.

In animals, Uehara et al. (2001) studied the effect of supplementing a diet with 5% fructooligosaccharides (FOS, a mixture of indigestible and fermentable sugars) for 7 d in Sprague-Dawley rats. Controls were fed the same diet without FOS. Isoflavones (40 mg or 148  $\mu\text{mol/kg BW}$ ) were given on d 5 of the feeding using a gastric tube. Plasma kinetics showed that in FOS-fed animals, genistein and daidzein concentrations from tail blood was significantly higher in the 48 h following FOS ingestion compared to controls and 24-48 h urinary genistein recovery was also higher in FOS-fed animals. Even though not significantly different, there was also a trend toward increased total 48 h urinary genistein excretion in FOS- compared to control-fed animals ( $14.5 \pm 1.2\%$  vs.  $11.6 \pm 1.5\%$  ingested dose). Urinary excretion of daidzein over 48h was similar in treatment and control groups ( $23.0 \pm 2.4\%$  vs.  $22.7 \pm 3.3\%$ ). This study shows that FOS modified and possibly delayed absorption of both daidzein and genistein. Despite similar amounts excreted in the urine over 48 h the proportion of isoflavone excreted in 0-24 h was higher in controls whereas 24-48 h excretion was higher in FOS fed animals. With a higher power (here,  $n=5-6$ ), 0-48h urinary genistein excretion values could also have been significantly different between treatments

Also in rats, Piskula (2000) looked at genistein plasma kinetics and conjugation in rats either fed or food deprived (24 h fasting,  $n=5/\text{group}$ , 7.9  $\mu\text{mol isoflavone/kg BW}$  injected using a gastric tube). In food-deprived rats, plasma daidzein and genistein reached a maximum concentration of  $20.9 \pm 4.4 \mu\text{mol/L}$  and  $11.4 \pm 3.1 \mu\text{mol/L}$ , respectively less than 10 min after ingestion). Fed rats reached a maximum plasma daidzein concentration of  $2.4 \pm 0.8 \mu\text{mol/L}$  2 h after ingestion while genistein plasma level reached  $1.8 \pm 0.2 \mu\text{mol/L}$  4 h after ingestion. Genistein was dissolved in  $\text{Na}_2\text{CO}_3$ , which increased gastric pH and improved absorption in fasted animals compared to fed animals that probably maintained a lower gastric pH due to digestion. More interestingly, the proportion of sulfate conjugates was significantly higher throughout the study in food-deprived rats, while glucuronide conjugates were dominant in fed rats. This phenomenon could be due to biotransformation activities in the liver that differ between fasted and fed states.

These two studies (Uehara et al. 2001; Piskula. 2000) showed that bioavailability in animals can be modified using dietary factors, such as insoluble fibers that may have an impact on gut transit time. Moreover, isoflavones ingested as part of a regular diet showed a different pattern of bioavailability compared to fasting. These findings deserve more investigation, with the goal of finding a factor that could improve significantly isoflavone bioavailability with relevance to humans, thereby enhancing possible health-promoting effects.

In humans, Tew et al. (1996) found that a high wheat fiber diet (40 g/d) had a negative impact on isoflavone bioavailability compared to a low fiber diet (15 g/d). Using a cross-over design and tofu or texturized vegetable protein (TVP) as sources of isoflavones, Tew et al. found that a high fiber diet decreased significantly plasma concentration (55% decrease at time 24 h;  $p < 0.05$ ) as well as urinary excretion (20% decrease;  $p < 0.03$ ) of genistein, which disagreed with Uehara et al. (2001) findings in animals. Different fiber diets may have different modes of action in modifying isoflavone bioavailability. On the other hand, Tew et al. did not find any difference in urinary recovery of daidzein or genistein expressed as % ingested dose from tofu or TVP, regardless of the fiber dose. Food matrix in this study did not influence overall apparent absorption.

Xu et al. (2000b) also concluded that food matrix or background diet did not influence isoflavone bioavailability. There was no difference in urinary excretion or plasma kinetics in subjects fed either a basic diet, a self-selected diet eaten at specific times or a self-selected diet eaten ad libitum. No difference was also observed when isoflavones were fed as part of tofu, tempeh, cooked soybeans or TVP.

With respect to gut transit time, Zheng et al. (2003) looked at in vitro disappearance of isoflavones and in vivo genistein urinary excretion and gut transit time in 35 Asian and 33 Caucasian females. Asians with low genistein in vitro fecal degradation had a significantly shorter mean gut transit time and greater genistein urinary excretion compared to Asians with rapid in vitro fecal genistein degradation (GTT was  $40 \pm 8$  h vs.  $63 \pm 5$  h and genistein urinary excretion was  $11.0 \pm 2.7\%$  vs.  $4.0 \pm 1.7\%$  of ingested dose from soymilk for low and high genistein degraders, respectively). This was not observed in Caucasian subjects, probably because Caucasian low genistein degraders had a mean gut transit time significantly

higher than Asian subjects with the same phenotype ( $86 \pm 10$  h vs.  $40 \pm 8$  h, respectively). Zheng concluded that a rapid gut transit time coupled with a low in vitro genistein disappearance resulted in greater bioavailability as reflected in urinary excretion.

#### **D. Identification of isoflavone-metabolizing and degrading microorganisms: use of genomic techniques**

Microbial ecology of a specific environment has been a challenge to assess both quantitatively and qualitatively. The numerous species present within the same environment creates such a complexity that techniques used over the past 30 years or more, such as plate count, has not given completely accurate data with regard to the original sample studied (Amman et al, 1995). New approaches to study microbial diversity were developed based on molecular biology techniques. One of them, namely polymerase chain reaction coupled with denaturing gradient gel electrophoresis (PCR-DGGE) was developed in 1993 by Muyzer et al. This technique was based on targeting and multiplying by PCR the DNA sequences coding for the V3 region of 16S rRNA with primers designed to match the conserved regions of the 16S rRNA gene. This sequence is different from one species to another and highly conserved within the same species. Consequently, the 16S rDNA sequence becomes an ID for the corresponding micro-organism. Theoretically, PCR products of the 16S rDNA sequences of a bacterial community should still represent the original population, both in a quantitative and a qualitative manner. In practice, this technique allows the amplification of all the major species present originally in the sample (species that represent 1% or more of the total community), but minor ones may not be represented accurately on the gel (Muyzer et al, 1993). DGGE allows the separation on an acrylamide gel done with a gradient of urea and formamide of the different PCR products sequences obtained based on their GC content and melting temperature ( $T_m$ ). During migration on the acrylamide gel, when a DNA sequence reaches its  $T_m$ , its helical structure partially melts, stopping the migration process through the gel (Muyzer and Smalla, 1998). Since PCR of fecal DNA creates a mixture of different DNA sequences, each one will stop at a different distance of migration, creating a banding pattern within the same lane. Depending on the primers used, the PCR products

varied in length from 200 to 400 bp (Fromin et al, 2002). Band patterns within the same lane or profile represent the bacterial composition of the sample studied. Muyzer et al. (1995) established that one band should represent only one sequence, which is consequently the representation of a single species. Moreover, the intensity of the band obtained after staining should represent the frequency of the species in the original sample studied (Murray et al, 1996). In other words, a band of strong intensity should represent a micro-organism present in greater quantity compare to a micro-organism that corresponds to a band of weak intensity. However, some studies have showed that within the same band, 2 sequences were found because of a strong similarity in their sequence and GC content (Vallaes et al, 1997). This raises the question of applying different gradients of acrylamide and different time of electrophoresis to obtain an optimal separation of the different PCR products (Muyzer and Smalla, 1998). Considering the possible pitfalls of PCR-DGGE (multiple species within the same band, presence of heteroduplexes and chimeric molecules (Ferris and Wards, 1997), inaccuracy in representing on the gel the original population diversity), it has been suggested that if all the samples are treated exactly the same way, one would assume that possible biases are normalized between samples, making the comparison of profiles and bands quite accurate (Fromin et al, 2002). On the other hand, advantages of PCR-DGGE include easiness, reproducibility, reliability and speed (Muyzer and Smalla. 1998). Indeed, considering the greater accuracy of this technique compare to standard microbiology techniques, such as plate count and sub-culturing of microbial samples, many studies have used PCR-DGGE to study microbial ecology from various samples. This technique can be applied to bacterial communities from soil and rhizosphere (Smalla et al. 2001), water (Murray et al. 1996), feces from animals (Simpson et al. 1999) or humans (Walter et al. 2001). Depending on the primers used, it also allows to study the microbial population as a whole, but primers specific for groups or families of bacteria have also been developed to study only a sub-set of the original samples. For example, Walter et al. (2001) used primers specific for lactic acid-producing bacteria of the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Weissella*, which is useful to assess the colonization of the colon by probiotics fed along with dairy products. PCR-DGGE becomes then an important tool in assessing the stability of a defined microflora over time or space (Muyzer and Smalla, 1998).

Any increase or decrease in bands intensity would then reflect greater or lower amounts of the corresponding micro-organisms, respectively. Depending on the outcome studied, one would focus on the whole profile or only on specific bands of interest.

With regard to gut microbial metabolism of isoflavones, the PCR-DGGE technique has not been applied yet, but could be useful in identifying the species responsible for metabolizing and degrading these compounds. Indeed, comparing gut microbial profile of subjects or animals with significantly different *in vitro* fecal degradation rates could lead to a correlation between degradation rates and intensity of one or more bands present among all profiles. In addition, variation in degradation rate within the same subject or animal over time could lead to compare profiles at different time points and assess changes in bands intensity that correlate with the change in degradation rate. Ultimately, the bands of interest correspond to bacterial species that could be involved in degrading isoflavones in the large intestine.

## **E. Human gut microorganisms**

### **1. Composition of the colonic/fecal microflora in humans**

Microflora of the large intestine is composed of a complex balance of bacterial strains needed for human health. Up to 500 different strains have been identified, but only 30-40 species are thought to compose more than 99% of this microflora. (table 2). (Hooper et al. 2002; Mai. 2004)

Most, if not all the strains identified in the human microflora are anaerobes. Facultative anaerobes include *Streptococcus*, *Enterococcus*, *Staphylococcus*, *Enterobacteria*, Yeast and *Lactobacillus*. Obligate anaerobes include *Bifidobacterium*, *Bacteroides*, *Fusobacterium*, *Eubacterium*, *Veillonella*, *Clostridium*, *Peptostreptococcus* and *Prevotella* Turner et al. (2003).



Bacteroides	Eubacteria	Bifidobacteria	Others
<i>B. fragilis</i>	<i>E. aerofaciens 1</i>	<i>B. adolescentis</i>	<i>Enterococcus faecium</i>
<i>B. vulgatus</i>	<i>E. aerofaciens 2</i>	<i>B. longum</i>	<i>Fusobacterium prausnitzii</i>
<i>bacteroides FB</i>	<i>E. rectale 1</i>	<i>B. infantis</i>	<i>Ruminococcus gnavus</i>
<i>B. thetaiotaomicron</i>	<i>E. rectale 2</i>		<i>Ruminococcus bromii</i>
<i>B. uniformis</i>	<i>E. bifforme</i>		<i>Ruminococcus obeum</i>
<i>Bacteroides FO</i>	<i>E. eligens</i>		<i>Ruminococcus albus</i>
<i>B. stercocis</i>	<i>E. lentum</i>		<i>Escherichia coli</i>
<i>B. caccae</i>			<i>Peptostreptococcus productus</i>
<i>B. distasonis</i>			

Table 2: Most prevalent species identified and cultured from human fecal sample (adapted from Finegold et al. 1974 and Moore and Moore. 1995)

Most of the studies about the gut microflora actually focused on analysis of fecal samples. To verify the physiological and microbiological relevance of the fecal ecology to the large intestine, Moore and Moore (1988) analyzed the bacterial composition of feces, ascending, transverse, descending colon and rectum of cadavers within 4 h of death. They found that microbial composition of the different segments of the large intestine were similar to feces, assuring then that fecal bacterial profile is of physiological relevance to the large intestinal physiology.

With special focus on ethnicity and diet, Moore and Moore (1995) analyzed the fecal flora from subjects of different ethnicities (Caucasian (n=17), Asian (n=22), African (n=16)). Regardless of ethnicities, they found that 371 taxa accounted for 96.7% of the total bacterial population, but 177 of these taxa were seen only once over analysis of 88 fecal samples, which meant that a limited number of taxa accounted for most of the fecal flora. The other 3.3% were assumed to be linked to minor taxa. They also found that *Eubacterium aerofaciens* type 1 was the most prevalent strain with 8.43% total bacterial population, followed by *Bacteroides vulgatus* at 7.79%. The bacterial composition with respect to ethnicities is described in the next section.

In the past 10 years, new techniques involving molecular biology have been developed to assess with more accuracy fecal bacterial profile and dynamics, both qualitatively and quantitatively. One of the techniques, PCR-DGGE, was reviewed in the previous section. Another variation of that technique involves the multiplication of 16S rDNA sequence coupled with cloning of the sequences using competent *E.coli* DHI cells. Using a moderate heat shock treatment, target DNA is inserted into *E.coli* cells as plasmids and multiplied using *E.coli* enzymatic and metabolic activities. DNA of colonies that contained a sequence were reamplified, enzymatically digested and sequenced. Using this technique along with more classical microbiology methods, like plate counts, Suau et al. (1999) looked at the 16S rDNA sequences obtained from fecal sample from one 40-year-old man. Microbiological techniques showed that 60-80% bacteria observed by staining could not be cultivated by classic bacterial culture techniques. Cloned 16S rDNA sequences showed 284 different clones that were grouped into 82 molecular species, themselves grouped into 3 phylogenetic groups: the *Bacteroides-Prevotella* group, the *Clostridium coccoides* group and the *Clostridium leptum* group. However, only 24% of the clones could be matched to sequences already known and available through genomic libraries. The *Bacteroides-Prevotella* group included several genres such as *Porphyromonas*, *Bacteroides* or *Prevotella*. *Clostridium coccoides* and *Clostridium leptum* groups included mostly *Clostridium*, *Ruminococcus* and *Eubacterium* species (See table 3). According to Suau et al. (1999), the species identified are the most prevalent ones in the human gut microflora and constitute up to 95% of total bacterial count. However, only 1 subject was screened and a higher power could have lead to the identification of a greater number of species. The fact that 76% of the clones matched with unidentified strains showed how complex the colonic microflora is and how only a little is actually understood about gut microbial identification, diversity and metabolism.

Harmsen et al. (2002) used a more extensive technique (dot blot hybridization and fluorescent in situ hybridization or FISH) with the development of 7 fluorescent probes, each specifically designed to match one group of bacteria: *Phascolarctobacterium* and relatives, *Veillonella*, *Eubacterium hallii* and relatives, *Lachnospira* and relatives, *Eubacterium cylindroides* and relatives and 2 probes for *Ruminococcus* and relatives. After incubation

with each of these probes, fluorescent bacteria were counted. These groups were chosen, because their 16S rDNA sequences are known and they are considered major groups of bacteria in the feces. Total bacterial counts among 7 subjects (ethnicity not mentioned) selected for that study ranged from  $8.6 \times 10^{10}$  to  $3.7 \times 10^{11}$  cells/g dry weight. In addition, there were major differences in bacterial counts of the different groups among the 7 subjects. For example, the *Eubacterium cylindroides* group, targeted to bind *E. cylindroides*, *biforme*, *tortuosum* and *dolichum*, *Clostridium innocuum* and *Streptococcus pleomorphus* varied from  $7.8 \times 10^7$  to  $9.3 \times 10^9$  cells/g dry weight. In addition, *Veillonella* group went undetected in 4 subjects up to  $4.3 \times 10^9$  cells/g dry weight in another subject. This study showed the great inter-individual microbial diversity among humans, but confirmed that many strains may indeed be found ubiquitously in all subjects.

Using the same FISH and specific probes technique Franks et al. (1998) looked at several bacterial groups: *Bacteroides fragilis*-*Bacteroides distasonis*, *Clostridium lituseburense*, *Clostridium histolyticum*, *Clostridium coccoides*-*Eubacterium rectale* and *Streptococci*-*Lactobacilli*. Bacterial counts using FISH showed again large variations from one subject to the other (n=7). For example, *Bacteroides* varied from  $3.1 \times 10^{10}$  to  $11.6 \times 10^{10}$  cells/g dry weight. Compare to bacterial staining and counting, 90-100% bacteria were identified and counted using FISH. Average bacterial count of *Bacteroides* was 20% of the total flora while the *Clostridium coccoides*-*Eubacterium rectale* group accounted for 29% of the total flora. Percentages of other groups were not given. In addition, subjects were followed over 8 months and the *Bacteroides fragilis* and *Bifidobacterium* groups showed the greatest inter- and intra-individual variability over time, while the *Clostridium coccoides*-*Eubacterium rectale* group showed greater stability. This study confirmed that *Bacteroides* and *Clostridium* are two of the major groups of the gut microflora. Inter-individual variability was also confirmed while intra-individual variability (without any apparent changes of diet or lifestyle) showed that some bacterial groups may be more unstable over time, thus questioning the actual overall stability of the gut microflora.

<b>Bacteroides-Prevotella group</b>	<b>Clostridium coccoides group</b>	<b>Clostridium leptum group</b>
<b>Porphyromonas</b> <i>P. salivosa</i> <i>P. catoniae</i> <i>P. gingivalis</i> <i>P. endodontalis</i>	<b>Clostridium</b> <i>C. herbivorans</i> <i>C. populeti</i> <i>C. coccoides</i> <i>C. nexile</i> <i>C. oroticum</i> <i>C. clostridiiforme</i> <i>C. celerecrescens</i> <i>C. xylanolyticum</i> <i>C. symbiosum</i> <i>C. aminovalericum</i> <i>C. aminophilum</i>	<b>Eubacterium</b> <i>E. siraeum</i> <i>E. plautii</i> <i>E. desmolans</i>
<b>Bacteroides</b> <i>B. putredinis</i> <i>B. splanchninus</i> <i>B. vulgatus</i> <i>B. fragilis</i> <i>B. stercoris</i> <i>B. uniformis</i> <i>B. eggerthii</i> <i>B. caccae</i> <i>B. ovatus</i> <i>B. thetaiotaomicron</i> <i>B. forsythus</i> <i>B. distatonis</i> <i>B. Merdae</i>	<b>Eubacterium</b> <i>E. xylanophilum</i> <i>E. ventriosum</i> <i>E. eligens</i> <i>E. formicigenerans</i> <i>E. contortum</i> <i>E. rectale</i> <i>E. hadrum</i> <i>E. halii</i> <i>E. ramulus</i>	<b>Ruminococci</b> <i>R. bromii</i> <i>R. flavefaciens</i>
<b>Prevotella</b> <i>P. pallens</i> <i>P. veroralis</i> <i>P. denticola</i> <i>P. oulora</i> <i>P. oris</i> <i>P. veroralis</i> <i>P. ruminicola</i>	<b>Ruminococcus</b> <i>R. obeum</i> <i>R. hansentii</i> <i>R. productus</i> <i>R. torques</i> <i>R. gnavus</i>	<b>Clostridium</b> <i>C. sporosphaeroides</i> <i>C. leptum</i> <i>C. viride</i>
		<b>Others</b> <i>Fusobacterium</i> <i>Termitobacter aceticus</i>

Table 3: Most prevalent species from the 3 major groups of bacteria identified in human fecal sample (adapted from Suau et al. (1999) and Fuller and Perdigon (2003))

As a summary of the data obtained from the different studies using FISH, Fuller and Perdigon (2003) reported inter-individual variability in bacterial groups from fecal samples of 15 healthy adult humans. Variations expressed as % of total fecal microflora were obtained using FISH:

<i>Bacteroides - Prevotella:</i>	1.5 – 96.3
<i>Bifidobacterium:</i>	0.4 – 44.6
<i>Atopobium-Eggerthella-Colinsella:</i>	0.1 – 23.0
<i>Eubacterium rectale-Clostridium coccoides:</i>	6.2 - 69.0
<i>Clostridium leptum:</i>	0.5 – 38.5

This study confirmed the results found by Harmsen et al. (2002) and Franks et al. (1998). Tremendous inter-individual variability was observed and the *Bacteroides/Prevotella* and *E. rectale/C. coccoides* are the two groups that reached the highest % of the total fecal flora, thus being the two most prevalent groups in the fecal flora. However, new groups, like *bifidobacterium* can account for a large % total flora. Precise quantification of bacterial groups is needed to produce elaborate and physiologically relevant data.

Finally, Matsuki et al. (2002) used a combination of culture of fecal suspension on non-specific anaerobic agar, DNA extraction of selected colonies and PCR with group-specific probes. The primers were designed for *B. fragilis*, *Prevotella*, *Bifidobacterium* and *C. coccoides* groups. Among the 6 subjects selected, *Prevotella* were only detected in two of them while the number of different strains of the *B. fragilis* group varied from 1 to 45, depending on the fecal sample studied. The major issue with this technique is that culturing fecal sample resulted in an average 54% decrease in bacterial count. Therefore, the number of isolated strains from one bacterial group may be biased by the loss of micro-organisms. Moreover, the techniques involving 16S rDNA and probes rely on known DNA sequences and corresponding micro-organisms. They give a more accurate estimate of the fecal bacterial profile compared to classic microbiological methods but identification of new strains cannot be done following this protocol.

## 2. Determinants of secondary metabolism of bacterial species

### - Bacterial metabolism and enzymatic activities

Depending on the efficacy of nutrient absorption in the small intestine, bacteria composing the gut microflora depend on the host's non-absorbed nutrients to survive and compete with other bacteria. The following list describes the types and quantities of nutrients arriving in the large intestine that bacteria can metabolize (From Fuller and Perdigon. 2003):

Resistant starch:	8-40 g/d
Non-starch polysaccharides:	8-18 g/d
Unabsorbed sugars:	2-10 g/d
Oligosaccharides:	2-8 g/d
Dietary protein:	3-9 g/d

In addition, these substrates are not metabolized the same way by micro-organisms that show a substrate-specificity so that certain end-products measured in the gut microflora reflect the presence of certain bacterial activities and bacterial groups. For example, with respect to carbohydrate metabolism, *Bacteroides* can produce acetate or propionate while butyrate formation can be linked to the presence of *Clostridium* or *Fusobacterium* (non-exhaustive list) (Fuller and Perdigon, 2003). The presence of bacterial groups can also be related to detection of other end-products of carbohydrate metabolism, such as ethanol, succinate, lactate, pyruvate or hydrogen.

### - Factors influencing the gut microbial composition

A few studies about factors affecting gut microbial profile have showed some interesting results. However, many of these studies looked at by-products or other endpoints of bacterial metabolism that reflect a change in the flora but they lacked a microbial analysis in identifying which actual strains are affected by these parameters. Below are a few studies that did look at changes in colonic ecology with respect to stress, ethnic background, diet, prebiotics and probiotics.

Holdeman et al. (1976) studied the fecal microflora of 3 astronauts during a 3-phase treatment: normal housing and normal diet, normal housing and Skylab diet (designed for food while in space), and confined housing and Skylab diet (stress period). Total microscopic counts and culture of the colonies using agar plates showed that the Skylab diet applied for 3.5 months did not have an effect on the gut microbial composition. However, the diet associated with confined housing provoked a rise in *Bacteroides fragilis subsp. thetaiotaomicron* (~3 to ~8.5 isolates/person/sample) while *Peptostreptococcus productus II* counts decreased significantly to almost no isolate found (5.3 to 1.5 isolates/person/sample). Results were normalized among subjects and fecal samples. This showed that stress (~2 months period), but not diet was a factor in modifying some of the major species of the gut microflora. However, technical difficulties to culture the microorganisms, the low power and particularity of the subjects and protocol made it difficult, even impossible to extrapolate to the general population. On the other hand, these early studies yielded some interesting hypotheses that could lead to more relevant data today, considering the enormous advances made to culture and analyze the gut microflora. Starvation, considered as a stress, has been studied mostly in animals (rats, mice, pigs) and all concluded that starvation significantly decreased lactobacilli counts (Tannock. 1997).

One of the early studies on diet and gut microflora was done by Finegold et al. (1974). They looked at 15 Japanese fed a traditional Japanese diet and 18 Caucasian fed a typical Western diet. Microbiologic counts and bacterial cultivation of microorganisms using selective media revealed that counts of *Streptococcus faecalis var. faecalis* (9.83 vs. 8.46 log<sub>10</sub> microorganisms/g feces), *Eubacterium contortum* (9.58 vs. 0), *Eubacterium lentum* (10.20 vs. 10.07) and *Peptostreptococcus sp. 1-25* (8.29 vs. 4.64) were significantly higher ( $p < 0.05$ ) in stool samples of Japanese vs. Caucasian, respectively. Only *Bifidobacterium infantis* was higher in Caucasian compare to Japanese (10.29 vs. 0 log<sub>10</sub> microorganisms/g feces). However, the species more prevalent in one ethnicity compare to the other were not studied for correlation to a specific food item. In the discussion, Finegold et al. also mentioned that due to diet (or ethnicity), *Clostridium* species are more prevalent in Japanese than Americans while the opposite may be true for *Fusobacterium* species. It is therefore

possible that diet do influence gut microbial profile and that long-term changes in dietary regimen could lead to significant changes in types and amounts of bacterial strains.

More recently Moore and Moore (1995) analyzed the fecal microflora from subjects of different ethnicities (Caucasian (n=17), Asian (n=22), African (n=16)). They found that, even though many of the bacterial strains were present among all ethnicities, their proportions varied tremendously (table 4 & 5).

This study showed that ethnicity, probably influenced by dietary factors as well as genetic background, is a major factor in the composition of the fecal/colonic flora. However, nutritional intervention to modify gut microbial profile in humans (short or long-term) have not been performed yet, so that diet associated with ethnicity could play an indirect role in determining the bacterial profile, but direct evidence is still lacking.

	Caucasians	Japanese	Africans
Total <i>Eubacterium</i>	24.6	11.3	28.1
Total <i>Bacteroides</i>	17.3	26.4	15.2
Total <i>Ruminococcus</i>	8.3	2.8	7.2
Total <i>Peptostreptococcus</i>	6.4	2.5	1.5
Total <i>Lactobacillus</i>	1.1	2.7	3.9
Total <i>Clostridium</i>	0	0	5.6
Total <i>Bifidobacterium</i>	4.8	5.5	3
Total <i>Fusobacterium</i>	3	3.3	4.7
Others	5.5	5.1	3.7

Table 4: Distribution of the major bacterial groups as % isolates in the fecal flora of healthy Caucasians on a western-type diet, rural native Japanese and rural native Africans. (Adapted from Moore and Moore. 1995).

Another way to modify the gut microbial profile is the introduction of probiotics (ingestion of living bacteria to improved gut health) and prebiotics (non-bacterial factor found to influence the gut microflora to improve health). Despite interesting finding about changes in specific strains in gut microflora (breast milk significantly increases *Bifidobacteria* population. Fuller and Perdigon. 2003), the effects of pre- and probiotics have been related to other endpoints that reflect a modification of the gut microflora, such as volatile fatty acids profile. On the other hand, significant changes in the gut profile as a



whole have not been fully investigated and/or understood yet. Table 6 is a non-exhaustive list of the major pre and probiotics used to improve gut health.

	Caucasians	Japanese	Africans
<i>Eubacterium aerofaciens</i> 1	8.5	7.5	15.2
<i>Eubacterium aerofaciens</i> 2	1.5	3.8	4.2
<i>Eubacterium rectale</i> 1&2	8.8		2.1
<i>Eubacterium rectale</i> 3	2.4		2.9
<i>Eubacterium biforme</i>	1.9		1.1
<i>Eubacterium eligens</i>	1.5		
<i>Eubacterium BN</i>			2.6
<i>Bacteroides vulgatus</i>	8.2	7.5	
<i>Bacteroides FB</i>		6	2.3
<i>Bacteroides thetaiotaomicron</i>	1.4	3.2	1
<i>Bacteroides fragilis</i>		2.5	8.7
<i>Bacteroides ovatus</i>		2.4	
<i>Bacteroides uniformis</i> 1&2	2.4	1.9	1.2
<i>Bacteroides stercoris</i>	1.6	1.5	
<i>Bacteroides FO</i>	1	1.4	
<i>Bacteroides caccae</i>	1.4		
<i>Bacteroides distasonis</i>	1.3		2
<i>Ruminococcus CE</i>	1.5		
<i>Ruminococcus gnavus</i>		1.5	
<i>Ruminococcus bromii</i>	5.2	1.3	3.9
<i>Ruminococcus obeum</i>	1.6		2.1
<i>Ruminococcus flavefaciens</i>			1.2
<i>Peptostreptococcus productus</i>	4.2	1.2	
<i>Peptostreptococcus DZ</i>	2.2	1.3	1.5
<i>Lactobacillus salivarius</i>		1.7	
<i>Lactobacillus acidophilus</i> 5	1.1	1	2.2
<i>Lactobacillus S06</i>			1.7
<i>Clostridium perfringens</i>			1.8
<i>Clostridium ramosum</i>			3.8
<i>Bifidobacterium adolescentis</i>	2.2	5.5	3
<i>Bifidobacterium longum</i>	2.6		
<i>Fusobacterium AB</i>			1
<i>Fusobacterium prausnitzii</i>	3	3.3	3.7
<i>Coprococcus comes</i>	1.4		
<i>Streptococcus SO3</i>		1.5	
<i>Enterococcus faecium</i>		3.6	
<i>Gemmiger formicilis</i>	1.6		1.4
<i>Escherichia coli</i>	1.2		2.3
<i>Butyrivibrio crossotus</i>	1.3		

Table 5: Distribution of the predominant species as % isolates in the fecal flora of healthy Caucasians on a western-type diet, rural native Japanese and rural native Africans. (Adapted from Moore and Moore. 1995).

<b>Prebiotics</b>	<b>Probiotics</b>
Lactulose	<i>Lactobacillus acidophilus</i>
Innulin	<i>Lactobacillus casei</i>
Fructooligosaccharides	<i>Lactobacillus bulgaricus</i>
Galactooligosaccharides	<i>Bifidobacterium adolescentis</i>
Lactosucrose	<i>Bifidobacterium bifidum</i>
Isomaltooligosaccharides	<i>Bifidobacterium breve</i>
Glucooligosaccharides	<i>Bifidobacterium longum</i>
Xylooligosaccharides	<i>Bifidobacterium infantis</i>

Table 6: Commonly used prebiotics and families of prebiotics and probiotics in humans. (From Fuller and Perdigon. 2003; Tannock. 1995).

### 3. Summary

Despite significant findings in the overall knowledge of gut microbial composition and metabolic activities, many aspects remain to be studied. Minor species that may vary among individuals have not been identified yet in spite of new accurate molecular biology techniques. Additionally, factors influencing composition of the gut microflora have been investigated from a metabolic point of view, but the actual changes in proportions of the different microbial species were not a research objective in these studies. Ethnicity-related gut microbial composition studied by Moore and Moore (1995) and Finegold et al. (1974) gave significant basis with relationship to diet for further studies, but long-term changes in dietary habits and how they would affect composition and metabolic activities of the colonic microflora remain until now a major knowledge gap that must be investigated in the near future. In addition, other factors supposedly influencing the gut microflora deserve more attention (gut transit time, sex and age).

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## ISOFLAVONE EXCRETION PHENOTYPES INFLUENCE PLASMA CHOLESTEROL IN GOLDEN SYRIAN HAMSTERS

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### ABSTRACT

We hypothesized that hamsters sorted into high and low isoflavone excreter phenotypic categories would show significant differences in plasma cholesterol status, the high isoflavone excreters having lower cholesterol. One-year old hamsters were fed either 1.18 mmol total isoflavones/kg diet (5 males, 5 females) or 1.77 mmol total isoflavones/kg diet (5 males, 4 females) for 10 d. Urine and feces were collected for 24 h in metabolic cages, and blood collected at euthanasia after 16h fast. According to a cluster analysis, hamsters sorted into two distinct urinary isoflavone phenotypes: high and low excreters with mean total isoflavone excretion of  $44 \pm 5\%$  v.  $14 \pm 7\%$ ; daidzein excretion of  $30 \pm 7\%$  v.  $8 \pm 3\%$ ; glycitein excretion of  $84 \pm 6\%$  v.  $28 \pm 13\%$  and genistein excretion of  $35 \pm 7\%$  v.  $8 \pm 4\%$  of the ingested dose, respectively. Urinary excretion of all isoflavones was strongly correlated ( $0.93 < r < 0.98$ ;  $p < 0.001$ ). Fasting plasma total cholesterol was significantly lower in high versus low excreters (e.g., based upon total isoflavones,  $6.1 \pm 1.9$  mmol/L v.  $8.3 \pm 1.5$  mmol/L, respectively), regardless of sex. Although females had significantly better bioavailability than males for total isoflavones ( $33 \pm 18\%$  v.  $17 \pm 9\%$ ), daidzein ( $24 \pm 14\%$  v.  $11 \pm 6\%$ ) and genistein ( $27 \pm 15\%$  v.  $10 \pm 6\%$ ), cholesterol concentrations were not significantly different by sex, probably because of the great interindividual variability in isoflavone bioavailability. These data support our hypothesis. Golden Syrian hamsters may be good models to study the link between bioavailability and cholesterol-lowering effects of isoflavones because they sort into isoflavone bioavailability clusters similar to what has been noted previously in humans.

Key words: isoflavone, hamsters, bioavailability, phenotypes, cholesterol.

Dietary isoflavones are found almost exclusively in soybeans and soy products (Murphy et al., 1997) and have an estrogen-like chemical structure (Setchell and Adlercreutz 1988). Evidence suggest that they may prevent chronic diseases such as breast cancer (Lamartiniere et al., 2002), but the health effects of these compounds are highly variable among individuals and their bioavailability is not well understood. In humans, 1 to 25% of the ingested dose of isoflavones is excreted in the urine, mostly as glucuronide conjugates, whereas less than 1% is recovered in the feces. The great proportion of isoflavones not recovered or disappearing has been linked with microbial metabolism and degradation (Hur et al. 2000; Xu et al. 1995). When isoflavones were incubated in vitro with human feces, they disappeared within 48 h. Fecal isoflavone degradation rates differed among individuals with degradation rates sorted into statistically significant clusters: high, moderate or low (Zheng et al. 2003). Low degraders seemed to have greater apparent absorption (urinary excretion) of isoflavones whereas high degraders had lesser urinary excretion (Xu et al. 1995). Zheng et al. (2003) found that Asian female low isoflavone degraders had more rapid gut transit time (GTT) and threefold greater urinary genistein excretion than did Asian high isoflavone degraders or Caucasian low or high isoflavone degraders. Caucasians had significantly longer GTT than did Asians.

Valid animal models need to be established to facilitate long-term study of dietary modulation of chronic diseases. In the case of cholesterol metabolism, Golden Syrian hamsters may be used to study the hypocholesterolemic effects of isoflavones, because cholesterol metabolism in hamsters is similar to that of humans (Bravo et al, 1994). Dorfman et al. (2003) found that the most appropriate protocol in using hamsters as a hypercholesterolemic model with relevance to humans was to feed Golden Syrian hamsters a diet containing 10% saturated fat and 0.1% cholesterol for 10 weeks. Song et al., (2003) showed that Golden Syrian hamsters fed 1.3 mmol daidzein/kg diet for 10 weeks had significantly lower concentrations of total cholesterol and non-HDL cholesterol compared with control hamsters fed a casein-based diet high in saturated fat. Isoflavone bioavailability has not been established yet in this animal model. Our hypotheses are that hamsters, like humans, cluster into significantly different isoflavone bioavailability phenotypes.



Furthermore, in hamsters these isoflavone bioavailability phenotypes correspond to the cholesterol-lowering efficacy of isoflavones.

## **MATERIALS AND METHODS**

### Chemicals and diets

Isoflavones were purchased as Novasoy® from ADM (Decatur, IL). Isoflavone composition was determined by HPLC (Murphy et al., 1997). Total isoflavone concentration was 1.18 mmol/g Novasoy® and daidzein, genistein and glycitein were 37%, 53% and 10% of total isoflavones respectively. The diet was high in saturated fat, casein-based with 10% sucrose in partial replacement of rice flour as a source of carbohydrates (Song et al., 2003). Diets were formulated to contain 1.18 mmol (320 mg) or 1.77 mmol (480mg) of total isoflavone/kg diet. Other chemicals used for isoflavone extraction and analysis were purchased from Fisher Scientific (Pittsburg, PA) and Sigma (St Louis, MO).

### Animals

The animal experimental protocol was approved by the Iowa State University Committee on Animal Care. Fifteen male and 14 female Golden Syrian hamsters (~12 weeks of age), were obtained from Harlan-Teklad (Madison, WI). They were housed individually in the same room in a temperature-controlled room (23°C) with a 12h light:dark cycle. Hamsters were maintained on a standard rodent chow diet until they reached 1 year of age. Males and females were randomly assigned to one of two diets containing either 1.18 mmol total isoflavone/kg diet (n=5 males, 5 females) or 1.77 mmol total isoflavone/kg diet (n=5 males, 4 females). Animals were fed ad libitum for 10 days and urine collected over 24 hours in metabolic cages. Urine was stored at -20°C until analysis. After 24h in metabolic cages, diets were withdrawn from hamsters 14-16h before euthanizing them under CO<sub>2</sub>. Blood was collected using cardiac puncture and plasma samples were obtained by centrifugation at 5000 g for 10 min at 4°C and frozen at -20°C until analysis.

### Urinary isoflavone analysis

Isoflavone extraction from urine samples was done following a modified protocol established by Zhang et al. (1999). Five mL of urine were incubated with 50  $\mu$ L of  $\beta$ -glucuronidase-sulfatase (Sigma, Saint Louis, MO) at 37°C for 20 h to obtain the aglycone form of isoflavones. Fifty  $\mu$ L of 2,4,4'-trihydroxydeoxybenzoin (THB) was also added prior to the incubation as an internal standard. Incubation mixtures were loaded on Extrelut (TM) QE columns (EM Science, Gibbstown, NJ) and extracted with ethyl acetate. The eluent obtained was collected, dried using a roto-evaporator (Buchi, Flawil, Switzerland) and dissolved in 9.8 mL of 20% ethanol and 0.2 mL of 1N HCL. Five milliliters of this solution was loaded on a pre-wetted Sep-pak C18 cartridge (Waters, Rainin, Woburn, MA), washed twice with 2mL of distilled deionized water and eluted with 2 mL of 80% methanol. Samples were stored at 4°C until HPLC analysis (Zhang et al., 1999). Urinary isoflavone excretion was expressed as a percentage of the dose ingested over 24 h, to normalize data across variations in food intake and body weight.

### Plasma total cholesterol analysis

Plasma total cholesterol analysis was performed using a standard diagnostic kit (Sigma Chem. Co., St Louis, MO). Each sample was measured in duplicate on a Beckman DU 500 spectrophotometer (Beckman Coulter, Fullerton, CA).

### Statistical analysis

Statistical analysis was performed using SAS (SAS Institute, version 6.12; 1998, Cary, NC). Phenotypes were established using a cluster analysis. Blood cholesterol and urinary excretion among the clusters, sexes and isoflavone doses were compared by one-way and two-way ANOVA and correlation between urinary excretion of isoflavones was done by a regression test. All results were considered significant at  $p < 0.05$ .

## RESULTS

### Urinary isoflavone excretion

Females fed 1.18 mmol/kg diet did not show any significant difference ( $p>0.05$ ) in urinary excretion of daidzein, genistein, glycitein and total isoflavone compared with the group fed 1.77 mmol/kg diet ( $32 \pm 21\%$  vs.  $35 \pm 17\%$ ;  $25 \pm 16\%$  vs.  $23 \pm 12\%$ ;  $57 \pm 37\%$  vs.  $69 \pm 33\%$  and  $25 \pm 18\%$  vs.  $29 \pm 14\%$  ingested dose for total isoflavone, daidzein, glycitein and genistein at 1.18 and 1.77mmol/kg diet, respectively). On the other hand, males fed 1.77 mmol/kg diet had significantly less urinary excretion of daidzein ( $16 \pm 6\%$  vs.  $7 \pm 3\%$ ;  $p=0.027$ ) and genistein ( $14 \pm 7\%$  vs.  $5 \pm 2\%$ ;  $p=0.024$ ) compared with males fed 1.18 mmol/kg diet (Table 1). Urinary excretion of total isoflavone and glycitein did not differ between the two doses in males ( $22 \pm 9\%$  vs.  $12 \pm 7\%$  and  $48 \pm 20\%$  vs.  $36 \pm 26\%$  ingested dose for total isoflavones and glycitein at 1.18 and 1.77 mmol/kg diet ( $p>0.05$ ), respectively).

Comparison of males and females fed the same dose of isoflavone showed that at 1.18 mmol/kg diet, no significant difference were found in urinary excretion of individual and total isoflavone ( $22 \pm 9$  vs.  $32 \pm 21$ ,  $p=0.37$ ;  $16 \pm 6$  vs.  $25 \pm 16$ ,  $p=0.28$ ;  $48 \pm 20$  vs.  $57 \pm 37$ ,  $p=0.66$ ;  $14 \pm 7$  vs.  $25 \pm 18$ ,  $p=0.23$  expressed % ingested dose for total isoflavone, daidzein, glycitein and genistein, of males vs. females respectively; table 1). On the other hand, a sex-difference was found between males and females fed 1.77mmol/kg diet in urinary excretion of total isoflavone ( $12 \pm 7$  vs.  $35 \pm 17\%$ ;  $p=0.03$ ), daidzein ( $7 \pm 3$  vs.  $23 \pm 11$ ;  $p=0.02$ ) and genistein ( $5 \pm 2$  vs.  $29 \pm 14\%$ ;  $p=0.008$ ). Glycitein urinary excretion was not significantly different ( $36 \pm 26$  vs.  $69 \pm 33\%$ ;  $p=0.08$ ) (table 1).

When sexes were combined, there was no significant effect of dose for daidzein ( $20 \pm 12$  vs.  $14 \pm 11$ ;  $p=0.32$ ), glycitein  $53 \pm 29$  vs.  $50 \pm 33$ ;  $p=0.87$ ), genistein ( $20 \pm 14$  vs.  $16 \pm 15$ ;  $p=0.55$ ), or total isoflavones  $27 \pm 16$  vs.  $23 \pm 17$ ;  $p=0.57$ ) (Table 1).

When doses were combined, females had significantly greater urinary excretion of total isoflavone ( $33 \pm 18\%$  vs.  $17 \pm 9\%$ ;  $p=0.026$ ), daidzein ( $24 \pm 14\%$  vs.  $11 \pm 6\%$ ;  $p=0.018$ ) and genistein ( $27 \pm 15\%$  vs.  $10 \pm 6\%$ ;  $p=0.005$ ) compared with males while glycitein was not statistically different between sexes ( $62 \pm 34\%$  vs.  $42 \pm 23\%$ ;  $p=0.14$ ) (Table 1).

Regardless of the dose of isoflavone fed or sex, glycitein bioavailability was significantly higher than that of daidzein ( $52 \pm 30\%$  vs.  $17 \pm 12\%$ ;  $p < 0.001$ ) and genistein ( $52 \pm 30\%$  vs.  $18 \pm 14\%$ ;  $p < 0.001$ ). Mean urinary excretion of daidzein and genistein were not significantly different ( $17 \pm 12\%$  vs.  $18 \pm 14\%$ ;  $p = 0.9$ ).

Urinary excretion of all animals, regardless of gender or dose, was ranked from the greatest to the least. Cluster analysis indicated the presence of high and low excreter (apparent absorber) phenotypes for all isoflavones. Mean urinary isoflavone excretion of low excreters was statistically different from that of the high excreters. Moreover, the range of urinary excretion showed large differences between the greatest and the least excreters. For example, female #2 fed 1.18 mmol total isoflavone/kg diet excreted only 4% of the ingested dose for total isoflavone whereas female #1 also fed 1.18 mmol total isoflavone/kg diet excreted 53% of the ingested dose for total isoflavone. Table 2 summarizes the range of excretion for each category of isoflavone; figure 1 gives an example of the different values and clusters obtained for total isoflavones (graphs not shown for daidzein, glycitein and genistein).

#### Correlation between urinary excretions of isoflavone

Excretions of each or total isoflavone were correlated, and all of these correlations showed an  $r$  value of 0.93 or greater. Moreover, regression analysis showed that all the correlations were statistically significant ( $p < 0.01$ ). Total isoflavone urinary excretion was significantly correlated with daidzein ( $r = 0.93$ ), glycitein ( $r = 0.97$ ) and genistein ( $r = 0.98$ ). In addition, daidzein urinary excretion showed a significant correlation with genistein ( $r = 0.97$ ) and glycitein ( $r = 0.98$ ) while genistein and glycitein urinary values were significantly correlated ( $r = 0.93$ ).

#### Plasma total cholesterol

Females fed 1.18 and 1.77 mmol/kg diet had similar cholesterol levels ( $7.7 \pm 0.9$  vs.  $5.7 \pm 1.8$  mmol/L;  $p = 0.19$ ). Similar results were found between males fed 1.18 and those fed 1.77 mmol/kg diet ( $7.5 \pm 2.2$  vs.  $8.8 \pm 1.1$  mmol/L;  $p = 0.29$ ). Comparison of mean plasma total cholesterol of animals fed the same dose of isoflavone did not show significant

differences between males and females ( $p=0.82$  and  $0.08$  for  $1.18$  and  $1.77$  mmol/kg males vs. females-fed animals, respectively) Despite sex differences observed in isoflavone urinary excretion, total cholesterol based only on sex did not show a significant difference in plasma total cholesterol ( $6.8 \pm 1.3$  vs.  $8.1 \pm 1.6$  mmol/L for male vs. females, respectively;  $p=0.18$ ) (Table 1).

Based on phenotypes of urinary excretion (regardless of sex or dose fed), mean plasma total cholesterol level of high excreters was significantly lower than that of low excreters for total isoflavones ( $6.1 \pm 1.9$  vs.  $8.3 \pm 1.5$  mmol/L;  $p=0.019$ ), daidzein ( $6.1 \pm 1.9$  vs.  $8.3 \pm 1.5$  mmol/L;  $p=0.019$ ) and genistein ( $6.3 \pm 1.5$  vs.  $8.4 \pm 2.0$  mmol/L;  $p=0.03$ ). Plasma cholesterol did not differ between glycitein excretion phenotypes ( $6.4 \pm 1.6$  vs.  $8.3 \pm 2.0$  mmol/L;  $p=0.054$ , Table 3).

## DISCUSSION

This study demonstrates that the clustering of individuals according to apparent isoflavone absorption as reflected in urinary excretion may predict the efficacy of isoflavone-containing products with respect to their cholesterol lowering ability. A hamster model may be useful in such studies because of similarities with humans in isoflavone bioavailability phenotypes.

Urinary excretion of the two isoflavone doses was not significantly different in females, whereas males fed  $1.77$  mmol total isoflavone/kg diet had lower bioavailability of daidzein ( $16 \pm 6\%$  vs.  $7 \pm 3\%$ ;  $p=0.027$ ) and genistein ( $14 \pm 7\%$  vs.  $5 \pm 2\%$ ;  $p=0.024$ ) than males fed  $1.18$  mmol total isoflavones/kg diet. Because the percentage urinary excretion is similar between the 2 doses fed to females but not males, we therefore concluded that a positive dose response occurred in females while males displayed a somewhat “inverse dose response”. Although this phenomenon has not been observed in Golden Syrian hamsters previously, a similar pattern has been observed by Janning et al. (2000) in female DA/Han rats fed by gavage a single dose of  $10$  or  $100$  mg daidzein/kg body weight (or  $37$  vs.  $370$   $\mu\text{mol/kg}$  body weight). Plasma area under the curve, another way to assess bioavailability of a component, was  $9.7\%$  and  $2.2\%$  for  $10$  and  $100$  mg dose, respectively. The authors

hypothesized some differences in kinetics related to the dose fed, but no explanation was provided. In the present study, the difference between the two doses fed (~118 vs. 177  $\mu\text{mol/kg}$  body weight, chronic intake) were not as great as in Janning et al. (2000) (37 vs. 370  $\mu\text{mol/kg}$  body weight of pure daidzein, acute intake) and it occurred only in males. In our study, although animals were distributed among groups at random, there may have been a greater proportion of low urinary excreters among the males fed the higher isoflavone dose, whereas high urinary excreters predominated among male fed the lower isoflavone dose. Randomization of females seemed to have produced a more even distribution of animals of each excretion phenotype in each dose fed, thus not observing a dose-related effect on urinary excretion. This unfortunate randomization of male animals could also explain the lack of difference in isoflavone excretion observed between males and females fed 1.18 mmol isoflavones/kg diet (Table 1), whereas significant sex difference in urinary isoflavone excretion occurred in animals fed 1.77 mmol isoflavones/kg (Table 1). This was also reinforced by analysis of gender and dose using a two-way ANOVA that did not give a significant p value (0.2).

With respect to sex differences observed here in animals, Lu et al (1998) observed in humans lesser isoflavone bioavailability in men than in women. During a one month intervention study, women (n=6) excreted 24 and 66% of the ingested doses of genistein and daidzein, respectively, whereas men (n=6) excreted 15 and 47% as genistein and daidzein respectively. Compared with Lu et al. (1998) study, male hamsters had urinary genistein excretion quite similar to men (15% and 10% of the ingested dose for men and male hamsters, respectively), but urinary excretion of daidzein was much less in male hamsters (47% and 11.8% for men and male hamsters, respectively). The same pattern of urinary excretion was also observed in women compared with female hamsters. Genistein levels were comparable (24% and 27.5% for women and female hamsters, respectively) but daidzein urinary excretion levels differed (66% and 24.5% for women and female hamsters, respectively). This showed that apparent absorption of genistein may be more similar between humans and hamsters compared with that of daidzein. When comparing different animal models, Bayer et al. (2001) investigated daidzein bioavailability in Fisher 244 male (n=4) and female (n=4) rats fed 100 mg daidzein/kg diet. Despite not finding sex

difference in urinary excretion as we did in this study, daidzein urinary excretion levels in rats were more similar to the ones found in the present study. Indeed, daidzein was excreted at  $8.8 \pm 3.1$  and  $8.2 \pm 2.8\%$  ingested dose for males and females respectively. Levels of daidzein metabolites in urine (ODMA and equol) accounted for less than 5% of ingested dose, so that the idea that daidzein is extensively converted into metabolites, especially equol, which would partly explain the low bioavailability of unchanged daidzein, was not confirmed in this study. In Janning et al. study (2000), plasma area under the curve was 9.7% and 2.2% for 10 and 100 mg daidzein, respectively in female DA/Han rats fed daidzein by gavage as a single dose and equol detection was so small that it was not accurately quantifiable. The possibility that that low daidzein bioavailability may be due to significant transformation of daidzein into equol is not supported by the data obtained from other animal studies. In the present study, we have not been able to detect the presence of equol in urine, possibly because the samples were partly diluted with drinking water when animals were in metabolic cages and only 5 mL were used for analysis. In addition, our limit of detection for equol using a UV detection method would not be likely to detect the seemingly small amounts of equol that may have been present. On the other hand, daidzein bioavailability data established in other rodent models confirmed the data obtained in our study. Janning et al. (2000) examined at plasma area under the curve, which indicated how much daidzein was absorbed over time, but it was still an indirect evidence of how much of the compound was likely to be excreted in the urine.

In humans, Lu et al. (1998) did not assess glycitein bioavailability, which was one of the main points of the present study. In humans, Zhang et al (1999; erratum, 2001) found that moderate degraders (n=14, 7 males and 7 females) had  $52.4 \pm 19.6\%$ ,  $37.0 \pm 25.9\%$  and  $47.6 \pm 18.1\%$  urinary recovery of the ingested dose of daidzein, genistein and glycitein, respectively. Urinary excretion of genistein was significantly less than that of daidzein and glycitein whereas urinary excretion of daidzein and glycitein were not statistically different.

These results taken altogether indicated that glycitein and daidzein were more absorbable than was genistein in humans. In the present hamster study, daidzein and genistein had similar patterns of urinary excretion (females showed 24% and 27% and males 11% and 10% for daidzein and genistein, respectively), whereas glycitein urinary recovery was higher (62%

and 42% for females and males, respectively). Urinary excretion of glycitein was greater and excretion of daidzein less in hamsters compared with humans who were moderate fecal isoflavone degraders (Zhang et al 1999). On the other hand, urinary excretion of genistein in hamsters who were high excretors was close to that of moderate human degraders. These differences may be due to a different gut microbial profile with presumably less glycitein-degrading and more daidzein-degrading microorganisms in hamsters compared with humans. We also hypothesized that sex differences could be due to a gut microbial adaptation over time in males that did not occur in females, but this remains to be identified specifically.

Phenotypes of bioavailability based on *in vitro* fecal isoflavone degradation have already been established in humans, who can be categorized as low, moderate or high isoflavone degraders (Zheng et al, 2003). In the present study we established our isoflavone bioavailability phenotypes based on urinary isoflavone excretion instead of *in vitro* fecal isoflavone degradation because we have not yet performed *in vitro* isoflavone incubations with hamster fecal or cecal contents. Zheng et al. (2003) showed that low urinary genistein excretion (and hence apparent absorption) corresponded with a high *in vitro* fecal degradation of genistein in Asian women, and this pattern was influenced by a significantly longer gut transit time. Considering that in the present study, genistein bioavailability in hamsters seemed to be more relevant to humans, it could then be considered as the trademark isoflavone when considering relationships between urinary, fecal excretion and *in vitro* degradation with relevance to humans. In order for hamsters to be a fully useful model to study bioavailability and health-promoting effects of isoflavones, it is crucial in future studies to evaluate the relationship between *in vitro* fecal degradation and urinary excretion of these compounds.

In previous studies (Zheng et al. 2003; Zheng et al 2004), a low bioavailability of daidzein tended to correspond with a low bioavailability of genistein and vice-versa. Correlation of daidzein or genistein with glycitein in humans has not been established yet, but unpublished data from our laboratory suggest that glycitein bioavailability was positively correlated with that of daidzein and genistein, although these correlations were not as strong as observed in hamsters. These data showed an additional common feature between hamsters and humans, namely that bioavailability phenotypes were generally consistent across the



different isoflavones. Indeed, there was a strong relationship among urinary excretion of all isoflavones so that a hamster being a high daidzein excreter was also a high genistein and glycitein excreter.

Because isoflavones are thought to be health-promoting compounds, we measured plasma total cholesterol as an endpoint in order to relate bioavailability to health. We chose plasma total cholesterol because lipid metabolism in Golden Syrian hamsters is close to that of humans and hypercholesterolemia can be induced in a short period of time in this animal model (Bravo et al, 1994). Our study showed that, regardless of sex and dose, total isoflavone, daidzein, genistein and to some extent glycitein had an effect on total cholesterol. Animals excreting low amounts of isoflavone had greater total cholesterol than those excreting large amounts. Greater isoflavone bioavailability was associated with a greater decrease in total cholesterol. This result is consistent with a previous study in hamsters showing the efficacy of daidzein in lowering cholesterol. Song et al. (2003) fed a high fat diet containing 1.3mmol/kg diet of purified daidzein to hamsters for 10 weeks, resulting in significantly lower levels of total cholesterol by ~24% and non-HDL cholesterol by ~35% in both sexes. There was no gender difference in improving blood lipid profile, which is in agreement with the present study. On the other hand, Lin et al. (2004) fed male hamsters (n=20/groups) for 5 weeks different diets containing either casein, plant sterol esters, soy isoflavones from Novasoy® or different combination of these four diet components. Hamsters fed isoflavones (~0.8 mmol total isoflavones/kg diet) did not differ from the casein control for total cholesterol, triglycerides or HDL-cholesterol. These results do not agree with the present study, but we fed both sexes higher doses of isoflavones than did Lin and colleagues, which suggest the possibility of a threshold dietary dose necessary to observe physiological effects. Lin et al. (2004) did not attempt to sort individuals according to isoflavone bioavailability. The great range of apparent isoflavone absorption (as reflected in excretion; from 2% of the ingested dose for genistein, up to 95% of the ingested dose for glycitein in the present study) could interfere with the effect of isoflavones on cholesterol metabolism. Thus, studies of health endpoints influenced by isoflavones should cluster individual endpoint variables relative to isoflavone bioavailability to appropriately relate to the health effect.

Human studies focusing on the hypocholesterolemic effects of isoflavones have also given variable results. Age, baseline blood lipid profile, isoflavone source (purified, dietary supplement or part of soy protein), dose of isoflavone and duration of the intervention varied from one study to another so that comparisons of protocols and results are difficult to make. According to Goodman-Gruen et al. (2001), one of the major disadvantages of providing soy protein is the variety and amounts of other bioactive compounds, such as saponins that might interfere with or mask isoflavone actions on cholesterol metabolism. It also seems that providing isoflavones to individuals with moderate to severe baseline hypercholesterolemia would produce more significant results in contrast to normocholesterolemic individuals. Crouse et al. (1999) studied the effect of isoflavones in moderately hypercholesterolemic men and women (n=156; LDL cholesterol levels 3.62 to 5.17 mmol/L). Subjects were divided into 5 groups (n=31) and given beverages for 9 weeks containing either 25 g casein/day, 25 g ethanol washed isolated soy protein/day that contained 3 mg total isoflavones or 25 g soy protein/day at three levels of isoflavones: 27, 37 or 62 mg total isoflavones. Subjects who consumed 62 mg isoflavones/day had significantly lower levels of total (4% reduction) and LDL cholesterol (6% reduction) compared with casein controls. In addition, subjects with baseline LDL in the highest range (>4.24 mmol/L) had a greater decrease in total (9%) and LDL cholesterol (10%) compared to casein. An 8% decrease was also observed in total and LDL-cholesterol of baseline-high LDL subjects given 37 mg isoflavones compare to casein group. Thus, this studied showed a threshold effect of isoflavones in order to improve blood lipid profile and more severely hypercholesterolemic subjects showed a greater benefit than did moderately hypercholesterolemic subjects. Dewell et al. (2002) gave 36 moderately hypercholesterolemic post-menopausal women (mean total cholesterol=  $6.6 \pm 1.3$  mmol/L) with 150 mg isoflavones/d (n=20) or placebo (n=16) for 2 mos. No significant differences were found in total and HDL-cholesterol over this time period in the isoflavone group. No differences were found between placebo and isoflavone groups in total and HDL cholesterol. The fact that studies of isoflavone efficacy vary significantly in their results suggests that screening for isoflavone bioavailability might permit more successful examination of the health effects associated with these compounds. Individuals with high urinary excretion/apparent absorption of isoflavones could have

significantly improved blood lipid profiles compared with those with lower urinary excretion/apparent absorption, as observed in hamsters.

The present study establishes the partial validity of Golden Syrian hamsters as an animal model to study bioavailability of isoflavones and their possible health-promoting effects. Great inter-individual variability in urinary excretion, grouping of animal in phenotypes of isoflavone bioavailability and strong correlations among types of isoflavones excreted are characteristics similar to what has been observed in humans. Our data, along with previously published data (Song et al. 2003; Crouse et al. 1999) showed benefits of isoflavones on cholesterol concentrations. On the other hand, differences in urinary excretion level of isoflavones between humans and hamsters are a major disadvantage. Glycitein was excreted in greater amounts in hamsters compared to humans, while daidzein showed an opposite pattern. Only genistein was similar between humans and hamsters. Based on these conclusions, health-related effects of genistein in Golden Syrian hamsters would then be relevant to humans, while more caution should be taken with respect to daidzein and glycitein when using of a rodent model. In addition, Golden Syrian hamsters could be considered as an animal model to study isoflavones bioavailability and their hypocholesterolemic effects with possible relevance to humans. Reproducibility of the results using both sexes with greater sample size, a single dose of isoflavones given and taking into account the role of variation in gut microbial ecologies would help in determining the observed hamster bioavailability phenotypes. This may provide useful insights into the effects of microbial/host interactions on chronic disease prevention.

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**Table 1** Mean isoflavone urinary excretion expressed as % ingested dose ( $\pm$  standard deviation) and corresponding mean plasma total cholesterol (mmol/L  $\pm$  standard deviation) in Golden Syrian hamsters, based on sex and/or dose of isoflavone.

	Isoflavone mmol/kg diet	Total isoflavone <sup>a</sup>	Daidzein <sup>a</sup>	Glycitein <sup>a</sup>	Genistein <sup>a</sup>	Plasma total cholesterol <sup>b</sup>
females (n=5)	1.18	32 $\pm$ 21	25 $\pm$ 16	57 $\pm$ 37	25 $\pm$ 18	7.7 $\pm$ 0.9
females (n=4)	1.77	35 $\pm$ 17	23 $\pm$ 12	69 $\pm$ 33	29 $\pm$ 14	5.7 $\pm$ 1.8
<b>p value</b>		<b>0.80</b>	<b>0.90</b>	<b>0.63</b>	<b>0.76</b>	<b>0.19</b>
males (n=5)	1.18	22 $\pm$ 9	16 $\pm$ 6	48 $\pm$ 20	14.6 $\pm$ 7.0	7.5 $\pm$ 2.2
males (n=5)	1.77	12 $\pm$ 7	7 $\pm$ 3	36 $\pm$ 26	5.5 $\pm$ 2.1	8.8 $\pm$ 1.1
<b>p value</b>		<b>0.11</b>	<b>0.02</b>	<b>0.42</b>	<b>0.02</b>	<b>0.29</b>
(n=10)	1.18	27 $\pm$ 16	20 $\pm$ 12	53 $\pm$ 29	20 $\pm$ 14	7.6 $\pm$ 1.5
(n=9)	1.77	23 $\pm$ 17	14 $\pm$ 11	50 $\pm$ 33	16 $\pm$ 15	7.4 $\pm$ 2.7
<b>p value</b>		<b>0.57</b>	<b>0.32</b>	<b>0.87</b>	<b>0.55</b>	<b>0.84</b>
males (n=5)	1.18	22 $\pm$ 9	16 $\pm$ 6	48 $\pm$ 20	14.6 $\pm$ 7.0	7.5 $\pm$ 2.2
females (n=5)	1.18	32 $\pm$ 21	25 $\pm$ 16	57 $\pm$ 37	25.9 $\pm$ 18.3	7.7 $\pm$ 0.9
<b>p value</b>		<b>0.37</b>	<b>0.28</b>	<b>0.66</b>	<b>0.23</b>	<b>0.82</b>
males (n=5)	1.77	12 $\pm$ 7	7 $\pm$ 3	36 $\pm$ 26	5 $\pm$ 2	8.8 $\pm$ 1.1
females (n=4)	1.77	35 $\pm$ 17	23 $\pm$ 12	69 $\pm$ 33	29 $\pm$ 14	5.7 $\pm$ 1.8
<b>p value</b>		<b>0.03</b>	<b>0.021</b>	<b>0.13</b>	<b>0.008</b>	<b>0.08</b>
females (n=9)		33 $\pm$ 18	24 $\pm$ 14	62 $\pm$ 34	27 $\pm$ 15	6.8 $\pm$ 1.3
males (n=10)		17 $\pm$ 9	11 $\pm$ 6	42 $\pm$ 23	10 $\pm$ 6	8.1 $\pm$ 1.6
<b>p value</b>		<b>0.02</b>	<b>0.01</b>	<b>0.14</b>	<b>0.01</b>	<b>0.18</b>

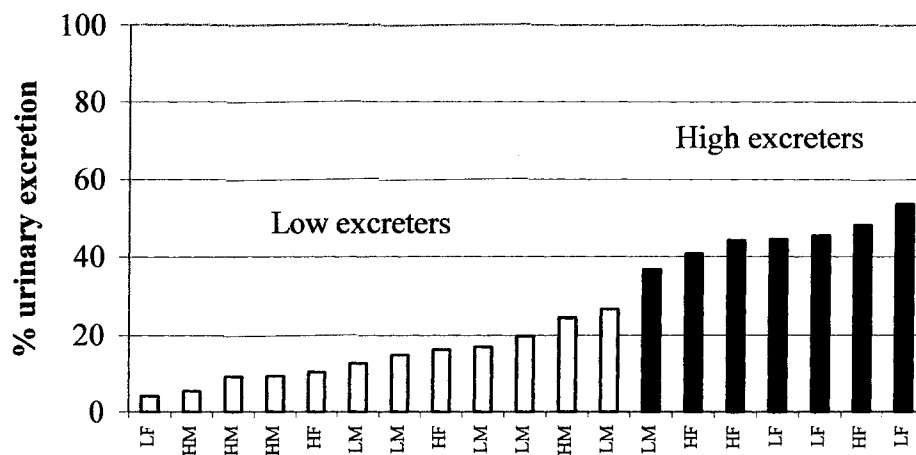
(a) Values are expressed as mean percentage of the dose ingested  $\pm$  standard deviation

(b) Values are expressed as mean plasma total cholesterol level (mmol/L)  $\pm$  standard deviation

**Figure 1** Individual values of urinary excretion of total isoflavone and clusters of high and low excreter phenotypes.

X-axis: Low dose (L), 1.18 mmol total isoflavone / kg diet; High dose (H): 1.77 mmol total isoflavone / kg diet; F: female; M: male.

White bars correspond to low urinary isoflavone excretors and black bars to high urinary isoflavone excretors.





**Table 2** Mean of urinary excretion of low and high excreter phenotypes and range of urinary excretion, regardless of sex or dose fed.

	Mean low excreter <sup>a</sup>	Mean high excreter <sup>a</sup>	Range of excretion <sup>b</sup>
Total	14.0 ± 7.1	44.8 ± 7.3	4.0 – 53.5
isoflavone			
Daidzein	8.6 ± 3.8	30.5 ± 7.0	2.9 – 39.9
Glycitein	28.7 ± 13.7	84.3 ± 6.8	8.9 – 95.6
Genistein	8.2 ± 4.6	35.6 ± 7.6	2.4 – 47.6

(a) Values are expressed as mean percentage of the dose ingested ± standard deviation

(b) Values are expressed as percentage of the dose ingested.

**Table 3** Plasma total cholesterol level of high versus low isoflavone excretors

	Total isoflavone	Daidzein	Glycitein	Genistein
High excretors	6.12 $\pm$ 1.99	6.12 $\pm$ 1.99	6.47 $\pm$ 1.66	6.35 $\pm$ 1.5
Low excretors	8.36 $\pm$ 1.56	8.36 $\pm$ 1.56	8.32 $\pm$ 2.08	8.4 $\pm$ 2.08
P value	0.019	0.019	0.054	0.03

Plasma total cholesterol levels are expressed in mmol/L  $\pm$  standard deviation.

**BIOAVAILABILITY OF SOY ISOFLAVONES IN GOLDEN SYRIAN HAMSTERS**

To be submitted to Journal of Nutrition

Mathieu Renouf and Suzanne Hendrich

**ABSTRACT**

Our hypothesis in this study was that Golden Syrian hamsters display similar patterns of bioavailability of isoflavones compared to humans in terms of apparent absorption, urinary excretion and gut microbial degradation. Two separate studies were conducted to test this hypothesis: one focusing on fecal (study #1) and the other on cecal (study #2) microbial degradation of isoflavones. Urinary excretion was significantly lower by 2-4 fold in males compared to females in both studies. Fecal isoflavone excretion was not significantly different between sexes or isoflavones (study #1) and showed low levels of excretion (<0.5% ingested dose). *In vitro* fecal degradation rates from study #1 showed low degradation levels and no significant correlation with urinary and fecal isoflavone excretion. However, cecal isoflavone degradation rates (study #2) were much higher than fecal isoflavone degradation rates (study #1) and were statistically correlated with urinary excretion of daidzein ( $r=0.90$ ;  $p=0.01$ ) and genistein ( $r=0.93$ ;  $p=0.004$ ) but not glycitein ( $r=0.50$ ;  $p=0.3$ ). Compared to published human data, female hamsters may be more relevant than males to study bioavailability of isoflavones, but strong sex differences deserve more attention. The lack of correlation and stability in glycitein bioavailability along with very little human data on this isoflavone suggest that more investigation is needed.

Key words: isoflavone, hamsters, bioavailability, cecal microflora.

Dietary isoflavones are found mainly in soybeans and soy products (Murphy et al., 1997) and have an estrogen-like structure (Setchell and Adlercreutz 1988). Evidence suggest that

they may prevent chronic diseases such as breast cancer in animal models (Lamartiniere et al., 2002), but the health effects of these compounds are highly variable among individuals and their bioavailability is not well understood. In humans, 1 to 25% of the ingested dose of isoflavones is excreted in the urine, mostly as glucuronide conjugates, whereas less than 1% is recovered in the feces. Women clustered into high and low urinary and fecal isoflavone excretors with different patterns of isoflavone bioavailability; high excretors had more similar availability of genistein and daidzein than did low excretors (Xu et al. 1995). The great proportion of isoflavones not recovered or disappearing has been linked with microbial metabolism and degradation (Hur et al. 2000; Xu et al. 1995). When isoflavones were incubated *in vitro* with human feces, they disappeared within 48h. Fecal isoflavone degradation rates differed among individuals. Degradation rates sorted into statistically significant clusters: high, moderate or low (Zheng et al. 2003). Zheng et al. (2003) found that Asian female low isoflavone degraders had more rapid gut transit time (GTT) and threefold greater urinary genistein excretion than did Asian high isoflavone degraders or Caucasian low or high isoflavone degraders; Caucasians had significantly longer GTT than did Asians.

Valid animal models need to be established to facilitate long-term study of dietary modulation of chronic diseases. Rats have been studied with respect to bioavailability of isoflavone (King et al. 1998). However, Golden Syrian hamsters deserve some attention because of the use of this animal model to study the hypocholesterolemic effect of isoflavones with relevance to humans. Bravo et al. (1994) showed that cholesterol metabolism in humans and hamsters are similar each other. Thus, to demonstrate even greater relevance, hamsters and humans would also have to share similarities in terms of apparent absorption, urinary excretion and gut microbial degradation. Our hypothesis in this study is that gut microbial disappearance of isoflavones in fecal or cecal samples from Golden Syrian hamsters predicts apparent absorption (urinary excretion) of isoflavones as previously shown in humans. To test this hypothesis, two separate studies were conducted: one focusing on fecal and the other on cecal microbial degradation of isoflavones.

## MATERIALS AND METHODS

### Chemicals and diets

Isoflavones were purchased as Novasoy® from ADM (Decatur, IL). Isoflavone composition was determined by HPLC (Murphy et al., 1997). Total isoflavone concentration was 963  $\mu\text{mol/kg}$  diet and daidzein, genistein and glycitein were 36%, 51.5% and 12.5% of total isoflavones respectively. The diet was formulated to contain 0.1% cholesterol and high level saturated fat from coconut oil (10%). The diet was casein-based as the main source of protein and formulated to mimic a typical Western-type diet (Song et al., 2003). Other chemicals used for isoflavone extraction and analysis were purchased from Fisher Scientific (Pittsburg, PA) and Sigma (St Louis, MO).

### Animals and study designs

The animal experimental protocols for both studies were approved by the Iowa State University Committee on Animal Care

Study #1: 20 males and 20 females Golden Syrian hamsters (~4 weeks of age) were obtained from Harlan-Teklad (Madison, WI). They were housed individually in the same room in a temperature-controlled room (23°C) with a 12 h light:dark cycle. Hamsters were maintained on a standard rodent chow diet for 2 weeks. Males and females were then fed ad libitum the isoflavone, casein-based diet for 2 weeks and put in metabolic cages for 24 h to collect urine and feces. Body weight and food intake were monitored every day until the end of the experiment. Urine analysis was performed within 48 h after collection and animals euthanized under CO<sub>2</sub> less than 48 h after isoflavone extraction and HPLC analysis of all urine samples. During that time, animals were fed the same isoflavone, casein-based diet, which was withdrawn 12 h before euthanizing them under CO<sub>2</sub>. Contents from the large intestine of the 4 highest and lowest urinary excreters were collected for *in vitro* fecal incubation.

Study #2: 10 males and 10 females Golden Syrian hamsters (~4 weeks of age) were obtained from Harlan-Teklad (Madison, WI). Protocols and design of this study were similar

to study #1, except that cecal instead of fecal contents from the three highest and lowest urinary excreters were collected for *in vitro* incubation. In addition, only urinary, but not fecal excretion analysis was performed in this study.

#### Urinary isoflavone analysis

Isoflavone extraction from urine samples was done following a modified protocol established by Zhang et al. (1999). Five mL of urine were incubated with 50  $\mu$ L of  $\beta$ -glucuronidase-sulfatase (Sigma, St Louis, MO) at 37°C for 20 h to obtain the aglycone form of isoflavones. Fifty  $\mu$ L of 2,4,4'-trihydroxydeoxybenzoin (THB) were also added prior to the incubation as an internal standard. Incubation mixtures were loaded on Extrelut (TM) QE columns (EM Science, Gibbstown, NJ) and extracted with ethyl acetate. The eluent obtained was collected, dried using a roto-evaporator (Buchi, Flawil, Switzerland) and dissolved in 9.8 mL of 20% ethanol and 0.2 mL of 1N HCL. Five milliliters of this solution was loaded on a pre-wetted Sep-pak C18 cartridge (Waters, Rainin, Woburn, MA), washed twice with 2 mL of distilled deionized water and eluted with 2 mL of 80% methanol. Samples were stored at 4°C until HPLC analysis (Zhang et al., 1999). Urinary isoflavone excretion was expressed as a percentage of the dose ingested over 24 h, to normalize data across variations in food intake and body weight. In both studies, urine analysis was performed within 48 h after collection in order to identify the highest and lowest urinary excreters for further colonic or cecal *in vitro* incubation.

#### Fecal isoflavone analysis

Fecal excretion of isoflavones was analyzed on 2 g of ground fecal sample mixed with 10 mL acetonitrile and 2 mL of 0.1 mol/L HCl. The mixture was stirred for 3 h, and then filtered through No. 1 Whatman filter paper. The eluent was collected in a round bottom flask and evaporated using a roto-evaporator (Buchi, Flawil, Switzerland). The dry residue was dissolved in 2 mL ethanol, 7.8 mL ultrapure water and 200  $\mu$ L of 1 Mol HCl. Five mL were loaded onto a pre-wetted Sep-Pak C18 cartridge (Waters, Rainin, Woburn, MA), washed with 2 mL of distilled deionized water and eluted with 2 mL of 80% methanol. Samples were stored at 4°C until HPLC analysis.

#### Fecal and cecal in vitro isoflavone analysis

At the end of each study, animals were euthanized under CO<sub>2</sub> and the content of the colon (study #1) or cecum (study #2) of the 3 or 4 highest and lowest urinary excreters placed in 15 mL of anaerobic brain heart infusion media or BHI (Fisher, Chicago, IL). BHI was prepared with 50 mL sodium bicarbonate/L media as a buffer and cysteine sulfide (Sigma, St Louis) as an oxygen indicator. After collection, contents were vortexed for ~15 sec to be homogenized and aliquoted by 5 mL volume in 20 mL of new anaerobic BHI media to which isoflavones were added to obtain a final concentration of 50 µM for each isoflavone. Isoflavones were synthesized chemically according to Chang et al. (1994) and Lang'at-Thoruwa et al. (2003). Duplicate tubes were prepared for each colonic or cecal sample obtained as well as a negative control (feces only). After adding isoflavone, each tube was then vortexed for 10 sec to homogenize feces and isoflavone and duplicate samples taken immediately afterwards and frozen at -60°C to measure isoflavone concentration at time 0. Tubes were placed at 37°C and isoflavones concentration over time was assessed by taking duplicate samples from each tube at time 3, 6, 9 and 12 h. All the samples taken were put at -60°C until isoflavone extraction and HPLC analysis. By flushing tubes with CO<sub>2</sub> and close them tightly, incubation tubes were maintained anaerobically during this process to preserve bacterial quality. To reduce isoflavone contamination in control fecal or cecal samples, diets were withdrawn ~12 h before euthanizing animals.

#### Statistical analysis

Statistical analysis was performed using SAS (SAS Institute, version 6.12; 1998, Cary, NC). Phenotypes were established using a cluster analysis. Fecal and urinary excretions among the clusters were compared by one-way ANOVA and correlations among the bioavailability data for the different isoflavones were done using linear correlation analysis. All results were considered significant at  $p < 0.05$ .

## RESULTS

### Body weight and food intake

Study #1: Initial mean body weights were similar ( $p=0.1$ ) between males ( $122 \pm 9$  g) and females ( $117 \pm 10$  g). Despite similar food intake between males and females ( $7.1 \pm 0.7$  g/d and  $7.3 \pm 0.5$  g/d, respectively;  $p=0.3$ ), females gained significantly more weight on a daily basis ( $1.0 \pm 0.3$  g/d) compared to males ( $0.7 \pm 0.2$  g/d;  $p=0.01$ ).

Study #2: Mean initial body weight was similar ( $p=0.09$ ) between males ( $114 \pm 4$  g) and females ( $119 \pm 7$  g). Food intake between male ( $7.6 \pm 0.5$  g/d) and females ( $7.7 \pm 0.4$  g/d) were similar ( $p=0.4$ ). Average daily body weight gain was statistically less ( $p=0.001$ ) in males ( $0.8 \pm 0.2$  g/d) than females ( $1.1 \pm 0.1$  g/d).

### Urinary isoflavone excretion

Study #1: Males and females differed greatly in urinary excretion of isoflavones (Figure 1). In males, daidzein and glycitein excretion were similar ( $18.2 \pm 7.8\%$  vs.  $15.8 \pm 6.3\%$  ingested dose;  $p=0.3$ ), while urinary excretion of genistein was significantly lower ( $7.8 \pm 6.2\%$  ingested dose) than that of daidzein ( $p<0.001$ ) or glycitein ( $p<0.001$ ). In females, mean glycitein and genistein urinary excretion were similar ( $31.4 \pm 11.2\%$  vs.  $26.7 \pm 11.5\%$  ingested dose;  $p=0.2$ ), while mean daidzein urinary excretion was significantly higher ( $44.2 \pm 13.7\%$  ingested dose) compared to that for glycitein ( $p=0.003$ ) and genistein ( $p<0.001$ ). Males were lower urinary excretors compared to females for daidzein ( $p<0.001$ ), glycitein ( $p<0.001$ ) and genistein ( $p<0.001$ ). Consequently, clustering of animals as high or low excretors based on their individual urinary isoflavone excretion values showed that females were mostly high excretors while males were mostly low excretors (Figure 3; data not shown for daidzein or glycitein). Regardless of sex, correlations between urinary excretions of the different isoflavones were all significant. Urinary daidzein correlated with both glycitein and genistein ( $r=0.97$  and  $r=0.96$ ;  $p<0.001$ ), as well as between glycitein and genistein ( $r=0.97$ ;  $p<0.001$ ).



Study #2: In males, daidzein and glycitein excretion were similar ( $13.6 \pm 7.6\%$  vs.  $11.0 \pm 5.1\%$  ingested dose;  $p=0.4$ ), while genistein urinary excretion was significantly lower ( $6.7 \pm 4.5\%$  ingested dose) than that of daidzein ( $p=0.03$ ) but not glycitein ( $p=0.07$ ). In females, mean glycitein and genistein urinary excretion were similar ( $18.2 \pm 8.0\%$  vs.  $15.8 \pm 9.4\%$  ingested dose;  $p=0.54$ ), while mean daidzein urinary excretion was significantly higher ( $29.6 \pm 13.4\%$  ingested dose) compared to glycitein ( $p=0.03$ ) and genistein ( $p=0.01$ ). Males were lower urinary excreters compare to females for daidzein ( $p=0.005$ ), glycitein ( $p=0.03$ ) and genistein ( $p=0.01$ ) (Figure 2). As observed in study 1, clustering of animals as high and low urinary excreters showed that females were phenotyped as high excreters while males were mostly low excreters (Figure 4; data not shown for daidzein or glycitein).

#### Fecal isoflavone excretion

In study #1, fecal isoflavone excretion was analyzed for each animal. Males and females showed less than 0.5% ingested dose present in the feces. There was no significant difference between isoflavones within and between sexes (Data not shown). Moreover, fecal excretion was not significantly correlated with urinary excretion for daidzein ( $r=0.35$ ;  $p=0.24$ ), glycitein ( $r=0.16$ ;  $p=0.6$ ) and genistein ( $r=0.29$ ;  $p=0.34$ ). However, daidzein fecal excretion correlated significantly with that of glycitein ( $r=0.92$ ;  $p<0.001$ ) and genistein ( $r=0.91$ ;  $p<0.001$ ), and glycitein with genistein ( $r=0.86$ ;  $p<0.001$ ).

#### Correlation between *in vitro* fecal degradation and urinary excretion of isoflavone

From study #1, based on their overall urinary excretion pattern for all three isoflavones, 4 high (3 females and 1 male, Fig. 3) and 4 low urinary excreters (3 males and 1 female, Fig. 3) were assessed for their *in vitro* fecal isoflavone degradation capacity. Mean degradation rates were not significantly different between high and low urinary excreters for daidzein ( $0.0101 \pm 0.0076$  vs.  $0.0151 \pm 0.0037 \text{ h}^{-1}$ ;  $p=0.28$ ), glycitein ( $0.0302 \pm 0.0178$  vs.  $0.0308 \pm 0.0357 \text{ h}^{-1}$ ;  $p=0.95$ ) and genistein ( $0.2655 \pm 0.1357$  vs.  $0.2978 \pm 0.1566 \text{ h}^{-1}$ ;  $p=0.76$ ). Correlations between *in vitro* fecal degradation rates and urinary excretion were not statistically significant for daidzein ( $r=0.49$ ;  $p=0.21$ ), glycitein ( $r=0.31$ ;  $p=0.44$ ) and genistein ( $r=0.33$ ;  $p=0.41$ ). Correlations between *in vitro* fecal degradation rates and fecal isoflavones excretion

were not statistically significant for daidzein ( $r=0.5$ ;  $p=0.19$ ), glycitein ( $r=0.28$ ;  $p=0.49$ ) or genistein ( $r=0.16$ ;  $p=0.69$ ).

#### Correlation between *in vitro* cecal degradation and urinary excretion of isoflavone

From study #2, based on their overall urinary excretion pattern for all three isoflavones, 3 high (2 females and 1 male, Fig. 4) and 3 low urinary excreters (3 males, Fig. 4) were assessed for their *in vitro* cecal isoflavone degradation capacity. Mean cecal degradation rates were significantly different between high and low urinary excreters for daidzein ( $0.031 \pm 0.015$  vs.  $0.123 \pm 0.029\text{h}^{-1}$ ;  $p=0.009$ ), and genistein ( $0.192 \pm 0.071$  vs.  $1.38 \pm 0.155\text{h}^{-1}$ ;  $p=0.004$ ) while mean glycitein degradation rates of high and low urinary excreters were not significantly different ( $0.186 \pm 0.096$  vs.  $0.310 \pm 0.099\text{h}^{-1}$ ;  $p=0.19$ ). Correlations between *in vitro* cecal degradation rates and urinary excretion were statistically significant for daidzein ( $r=0.90$ ;  $p=0.01$ ) and genistein ( $r=0.93$ ;  $p=0.004$ ; Figure 5; data not showed for daidzein and glycitein), but failed to show a significant correlation for glycitein ( $r=0.50$ ;  $p=0.3$ ).

## **DISCUSSION**

This study focused on establishing Golden Syrian hamsters as a potential animal model to study bioavailability with relevance to humans.

Despite initial body weight and similar daily food intake, females gained significantly more weight on a daily basis compared to males. This fits typical growth patterns for hamsters. Urinary isoflavone excretion levels showed a similar pattern between studies in females (daidzein>glycitein=genistein). In males, glycitein urinary excretion in males shifted from being similar to daidzein and significantly higher than genistein in study 1 to being not different from either other isoflavone in study 2, suggesting that glycitein bioavailability in males may be more unstable than in females. However, aside from glycitein, daidzein apparent absorption was greater than that of genistein in both sexes and both studies. A similar pattern was also observed in humans across several bioavailability studies (Xu et al. 1994 & 1995; Lu et al. 1998; Zheng et al. 1999; erratum 2001). Our data also differed from Lee (2004), a study in which female hamsters (10/group; 11-12 weeks old) were fed a single

purified isoflavone in the diet for 4 weeks. They found mean urinary excretion levels of  $4.6 \pm 1.8$ ,  $32.2 \pm 25.7$  and  $2.2 \pm 1.7\%$  for daidzein, glycitein and genistein, respectively. Thus, feeding one versus all three isoflavone together led to less daidzein and genistein excretion compared with that of glycitein. This might be partly due to the absence of competition among isoflavones for microbial degradation so that daidzein and genistein were much more degraded. Other differences possibly responsible for these data were age of animals (12 weeks vs. 6 weeks in the present study) and greater equimolar concentration of dietary isoflavones in the diet ( $\sim 50 \mu\text{mol/kg}$  body weight/day). In our study, animals were fed 25, 8 and  $35 \mu\text{mol/kg}$  body weight/day of daidzein, glycitein and genistein, respectively. Dose, age, sex and proportion of isoflavones when fed together would therefore be significant factors to consider for future studies.

A strong sex difference was observed in both studies, with males being lower urinary excretors of all three isoflavones compared to females. With respect to humans, Lu et al (1998) also observed lesser isoflavone bioavailability in men than in women. During a one month intervention, women ( $n=6$ ) excreted 24 and 66% of the ingested doses of genistein and daidzein, respectively, whereas men ( $n=6$ ) excreted 15 and 47% as genistein and daidzein respectively. Lu et al. (1998) findings suggested that in humans, as in hamsters, males are lower urinary excretors than females. While urinary levels detected by Lu et al. (1998) in women were within the same range observed in the present study with respect to female hamsters for daidzein ( $\sim 55$  vs. 40% ingested dose) and genistein ( $\sim 20$  vs.  $\sim 20\%$  ingested dose for Lu and present study, respectively), levels between men and males hamsters were different for daidzein ( $\sim 15$  vs.  $\sim 45\%$  ingested dose) and genistein ( $\sim 8$  vs.  $\sim 15\%$  ingested dose). Thus, despite some discrepancies between studies in female hamsters, overall levels of urinary excretion showed greater similarities to humans than did male hamsters. On the other hand, the present data in males confirmed those obtained in other rodent models. King et al. (1998) compared the bioavailability of daidzein and genistein in male Wistar rats fed a soy extract providing  $74 \mu\text{mol}$  genistein and  $77 \mu\text{mol}$  daidzein/kg body weight. 48 h urinary excretion of daidzein and genistein were  $17.4 \pm 1.2\%$  ( $\sim 15\%$  in the present study) and  $11.9 \pm 1.1\%$  ( $\sim 8\%$  here) of the ingested dose, respectively, while fecal excretion was  $2.3 \pm 0.5\%$  and  $3.4 \pm 0.4\%$ , respectively. Thus, male rodents may not be the best model to study

bioavailability of isoflavones considering a much lower ability to excrete (apparent absorption) isoflavones whereas females may be overall more suitable overall for data extrapolation to humans.

Fecal isoflavone excretion in study #1 did not show any sex difference and very low levels of excretion (0.1 to 0.5% ingested dose). King et al. (1998) found up to 3.5% ingested dose present in fecal samples from rats, while in humans, Xu et al. (1995) found that subjects who excreted large amounts of isoflavone in their urine also excreted larger amounts in their feces (up to 6% ingested dose) compared to lower urinary excreters. Thus, since female hamsters were significantly higher urinary excreters than males, we expected greater amounts of isoflavones in feces from females than males. No significant difference was found, neither was there a correlation between fecal and urinary excretion of individual isoflavones. Xu et al. (1995) and Zhang et al. (1999; erratum 2001) both found positive correlations between isoflavone excretion in urine and feces. We therefore hypothesized that fecal microbial activity in hamsters may be greater than in humans or other rodent models, thereby explaining such low levels of fecal excretion. In addition, sex differences in microbial degradation and possibly biliary excretion of isoflavones being greater in males than females could explain the low levels of urinary excretion obtained in male hamsters.

To predict apparent absorption and pattern of urinary excretion in humans, *in vitro* fecal degradation of isoflavones has shown applicability in determining bioavailability of isoflavones in humans (Hendrich et al. 1998; Xu et al. 1995; Zhang et al. 1999; erratum 2001). Indeed, subjects who were high fecal degraders *in vitro* were low urinary and fecal excreters while those who degraded smaller amounts of isoflavones *in vitro* excreted greater proportion of the ingested dose in both urine and feces. Thus we originally hypothesized that fecal *in vitro* degradation rates in hamsters would correlate with urinary excretion patterns. However, fresh feces collected from the large intestine from high and low urinary isoflavone excreters after euthanizing them failed not only to show any correlation to urinary or fecal excretion, but also the range of isoflavone degradation rates obtained were very low. Degradation rates of low urinary excreters (hypothesized high *in vitro* degraders) did not exceed 0.015, 0.030 and 0.300h<sup>-1</sup> for daidzein, glycitein and genistein respectively, while in humans, high fecal isoflavone degradation rates reached 0.299 and 0.400h<sup>-1</sup> for daidzein and

genistein, respectively (Hendrich et al. 1998). Only genistein degradation seemed to reach levels that showed some fecal bacterial activity, but the absence of correlation with urinary excretion and low to nearly absent fecal degradation of daidzein and glycitein led us to the conclusion that fecal *in vitro* degradation and the fecal microflora were not a useful tool to assess bioavailability of isoflavones in this animal model and that hamster fecal isoflavone degradation might not be relevant to human fecal isoflavone degradation. Investigation of cecal degradation rates in study #2 produced more appropriate data. Indeed, high daidzein and genistein degraders had a mean degradation of 0.123 and 1.38h<sup>-1</sup>, which showed greater bacterial activity and degradation of isoflavone compared to fecal activity. Significant correlation between urinary excretion of isoflavone and their associated *in vitro* cecal degradation rates of daidzein and genistein was of greater biological similarity to human fecal isoflavone degradation rates. Therefore, the cecal microflora was more active than the fecal microflora with respect to isoflavone degradation in hamsters and shown to be of greater relevance to the fecal microflora in humans when depicting and comparing the overall bioavailability pattern in humans versus hamsters. Hence, hamster cecum is more metabolically equivalent to the human colon. Glycitein remained an issue in the present study considering that neither fecal nor cecal degradation correlated significantly with urinary excretion. The absence of conclusive human fecal degradation data on glycitein and its relationship to isoflavone bioavailability did not allow us to draw any definite conclusions on the data obtained in the present study. Further investigations in both models are needed to understand with greater accuracy bioavailability of glycitein.

## CONCLUSION

The present study investigated in more details the bioavailability of isoflavones and how relevant the hamster model may be to humans. The main reason for this investigation was that if hamsters and humans share similar patterns of bioavailability of isoflavones, this animal model could then be used to study prevention of chronic diseases that could not be induced in humans (hypercholesterolemia, cancer...). From both studies, we concluded that female hamsters had a more similar pattern of urinary excretion compared to women, while

male hamsters had a lower isoflavone bioavailability than men. However, a strong and constant sex difference was observed across both studies and was relevant to humans. We also predicted that a sample size of at least 20 animals/ group would produce more robust bioavailability patterns of all three isoflavones. Moreover, hamster cecal but not fecal bacterial activity was strongly related to urinary excretion of daidzein and genistein as observed in humans between *in vitro* fecal degradation and urinary excretion. Therefore, the hamster cecal microflora is hypothesized to be metabolically analogous to the human fecal microflora. Further investigation of glycitein metabolism and bioavailability in both hamsters and humans is needed.

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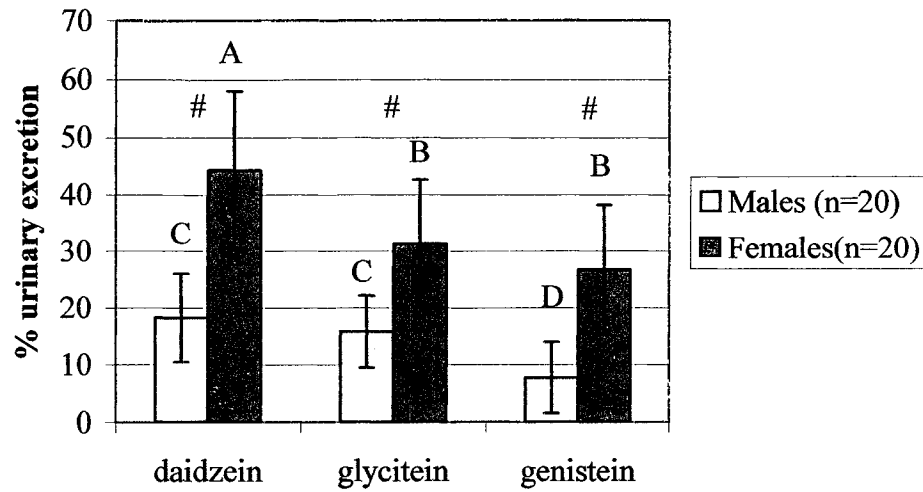
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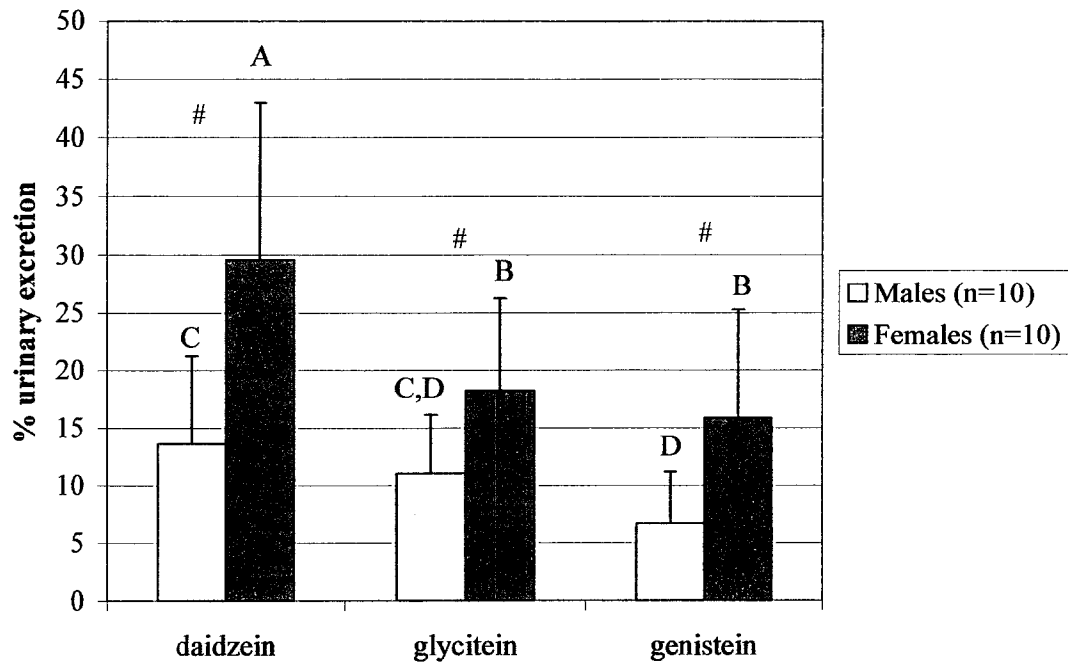
Figure 1: Comparison of mean percentage urinary excretion of ingested dose between male and female Golden Syrian hamsters (Study 1).



Different letters indicate statistically significant differences across sexes and isoflavone.

# p value < 0.05: indicates statistical difference between males and females

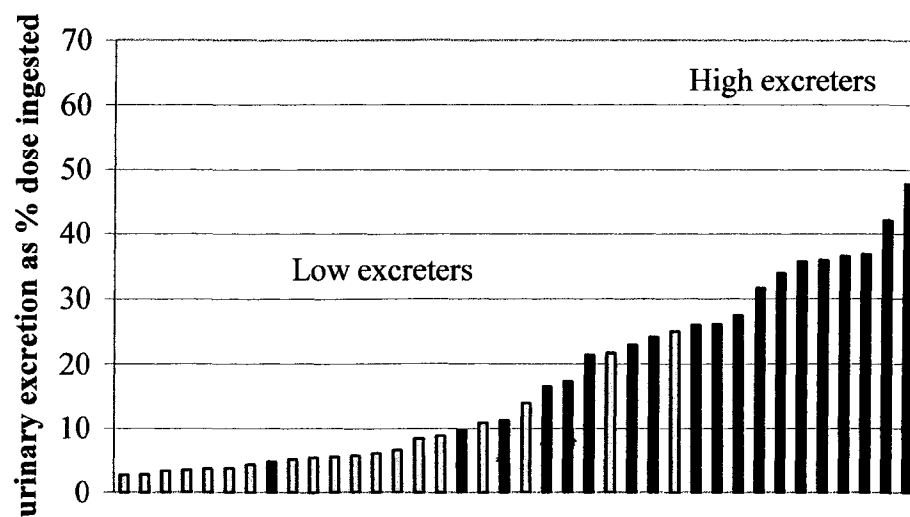
Figure 2: Comparison of mean percentage urinary excretion of ingested dose between male and female Golden Syrian hamsters (Study 2).



Different letters indicate statistically significant differences across sexes and isoflavone.

# p value < 0.05: indicates statistical difference between males and females

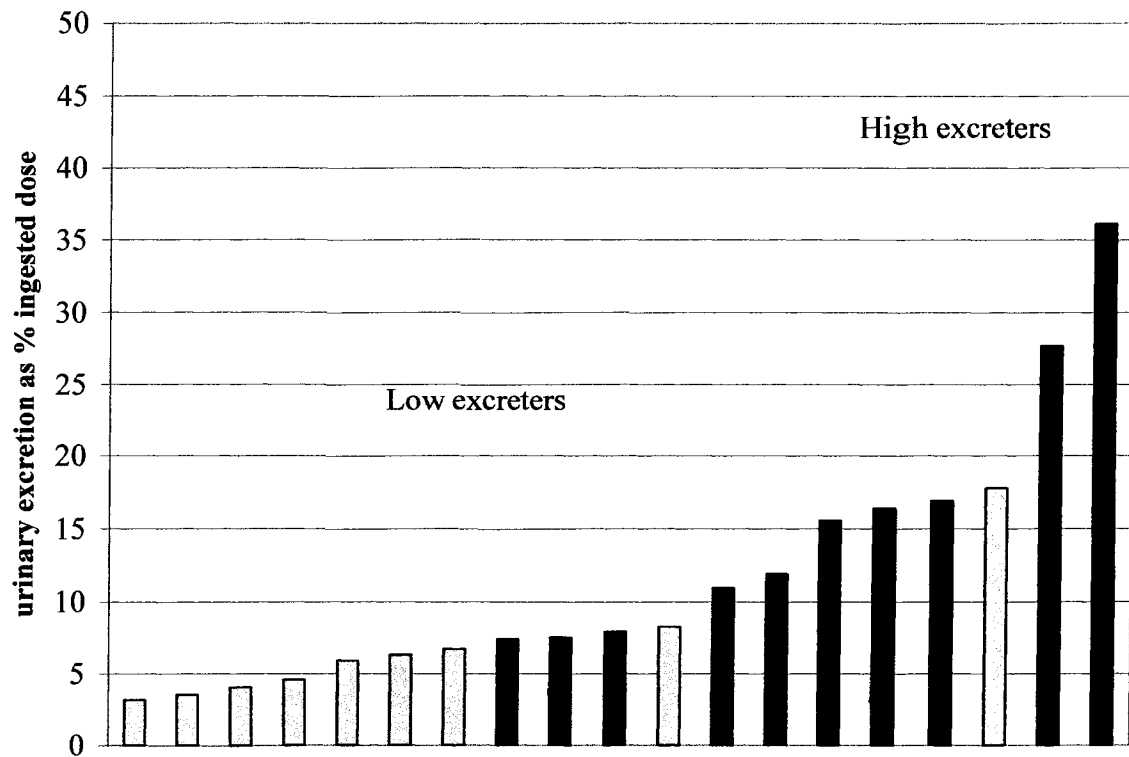
Figure 3: Ranking and cluster analysis of individual genistein urinary excretion values in male and female Golden Syrian hamsters (Study #1)



Grey bars are male hamsters

Black bars are female hamsters

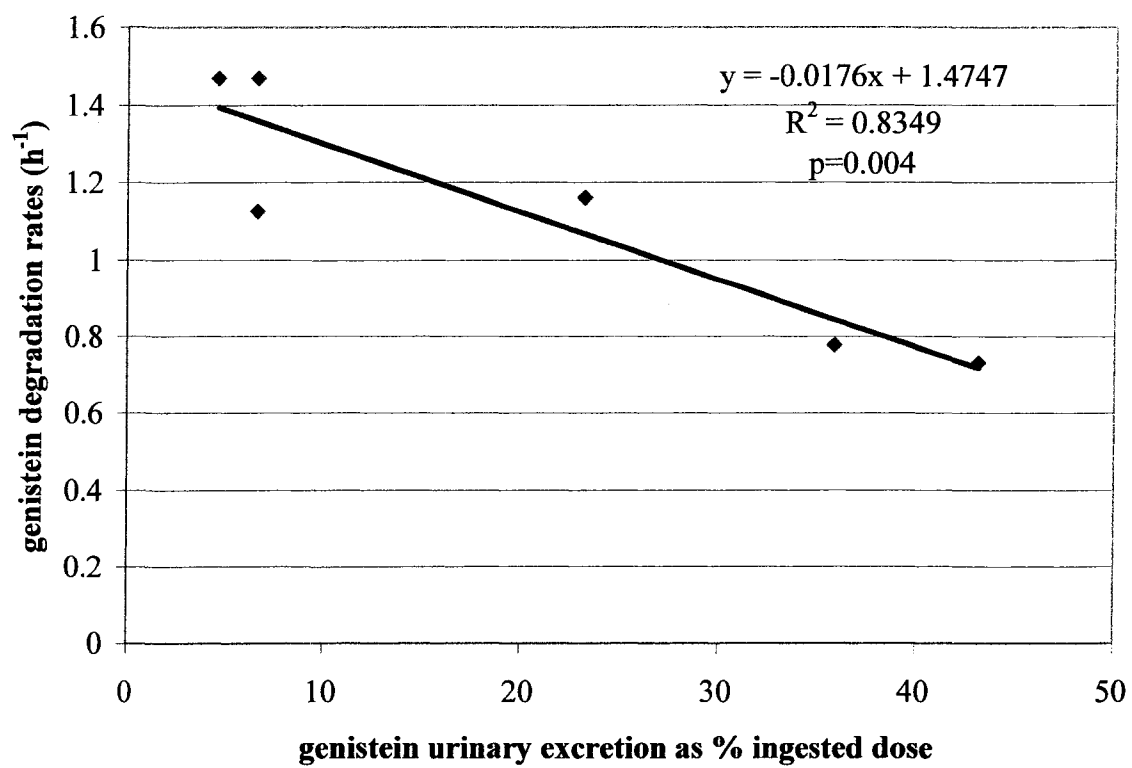
Figure 4: Ranking and cluster analysis of individual genistein urinary excretion values in male and female Golden Syrian hamsters (Study #2)



Grey bars are male hamsters

Black bars are female hamsters

Figure 5: Correlation between genistein in vitro cecal degradation rates and in vivo urinary excretion data between high (n=3) and low (n=3) urinary excretors (Study #2).



**IDENTIFICATION OF *BACTEROIDES OVATUS*, *BACTEROIDES ACIDIFACIENS*,  
*EUBACTERIUM RAMULUS*, *CLOSTRIDIUM ORBISCINDENS* AND *TANNERELLA*  
*FORSYTHENSIS* AS MICROORGANISMS INCREASING ISOFLAVONE  
DISAPPEARANCE IN HUMAN FECES, USING POLYMERASE CHAIN  
REACTION AND DENATURING GRADIENT GEL ELECTROPHORESIS**

Manuscript in process.

Mathieu Renouf, Andrean L. Simons and Suzanne Hendrich

**ABSTRACT**

Subjects who apparently absorb small amounts of isoflavones show high *in vitro* fecal degradation of these compounds. We hypothesized that high fecal isoflavone degradation rate coincided with distinct fecal bacterial species. To identify these bacteria, fresh feces from 33 healthy adult subjects (20 men, 13 women, mean age  $26 \pm 4$  years; 15 Caucasians, 8 Indians, 6 Asians and 4 African Americans) were incubated anaerobically with isoflavones to assess degradation rates using HPLC. Fecal DNA was extracted, bacterial 16S rDNA sequences amplified by polymerase chain reaction (PCR) and separated by denaturing gradient gel electrophoresis (DGGE). Cluster analysis identified high and low degraders of daidzein, genistein and glycitein. DGGE analysis showed that high genistein degraders ( $n=4$ ; fecal degradation rate  $1.47 \pm 0.14 \text{ h}^{-1}$ ) shared 5 bands of greater intensity than found in feces of low genistein degraders ( $n=4$ ; fecal degradation rate  $0.146 \pm 0.034 \text{ h}^{-1}$ ) high glycitein degraders ( $n=4$ ;  $0.574 \pm 0.299 \text{ h}^{-1}$ ) also shared 5 bands of greater intensity than found in feces of low glycitein degraders ( $n=4$ ;  $0.146 \pm 0.034 \text{ h}^{-1}$ ).

Sequencing of 16S rDNA from the bands of interest showed concordance with known species from the *Bacteroides* and *Prevotella* genus as well as the Clostridiales order. In one individual who switched from high to low glycitein degradation rate when fecal samples were analyzed at two time points one month apart, 5 DNA bands associated with high glycitein degradation were matched to bacteria from the same bacterial families as those

identified in the other subjects who were high glycitein and/or genistein degraders. After developing two *in vitro* systems, one rich (rumen fluid based brain heart infusion media) and one poor in nutrients (feces incubated overnight in brain heart infusion media), we identified *Bacteroides ovatus*, *Bacteroides acidifaciens*, *Eubacterium ramulus*, *Clostridium orbiscindens* and *Tannerella forsythensis* as the major human gut microbial species that increased isoflavones degradation compare to control under both nutrient rich and poor conditions, thus the presence of these species may indicate the greatest relevance in degrading isoflavone in the human gut. Significant increases in isoflavone disappearance with other species only in nutrient-poor conditions suggested that some species may influence isoflavone degradation when nutrient availability is lower. These species showed different response in increasing isoflavone degradation from one individual to another and from isoflavone to another between and within individuals, demonstrating the complexity of bacterial activity with respect to isoflavone degradation. Bacterial species that stimulated isoflavone degradation in nutrient rich media are likely to be the major species involved in isoflavone degradation in both high and low degraders. We propose that the greater amounts of these species found in high degraders may predict lesser overall bioavailability of isoflavones in such individuals. Microbial species influencing isoflavone degradation in nutrient poor conditions only seem to be the major species accounting for the high fecal genistein or glycitein degradation phenotype in humans. Intersubject variability in fecal isoflavone degradation rate was useful in identifying human gut microbial species associated with isoflavone degradation and will deserve further attention.

**Key words:** PCR, DGGE, fecal microflora, isoflavone-degrading microorganisms, bioavailability.

Isoflavones are found in the human diet almost exclusively in soybeans and soy products. Their chemical structure is estrogen-like (Setchell and Adlercreutz 1988). Moreover, there has been evidence that isoflavones consumption may prevent several chronic diseases, such as breast cancer where higher soy consumption (>18.66 nmol isoflavone/mg creatine in urine) was associated with lower breast cancer risks compare to lower soy

consumption ( $<5.58$  nmol/mg creatine in urine) (Zheng et al. 1999), but the health effects of these compounds are highly variable among individuals, which may be attributed in part to variability in bioavailability. One to 25% of the ingested dose of isoflavones is excreted in the urine, while less than 1% is recovered in the feces. The great proportion not recovered or disappearing has been linked to microbial metabolism, where fresh but not autoclaved feces were capable of degrading isoflavones over time (Xu et al. 1995; Hur et al. 2000; Zheng et al. 2003 & 2004). When isoflavones are incubated *in vitro* with feces, they disappear almost completely within 48 h. Fecal isoflavone degradation rates can be statistically sorted into clusters so that people are grouped as high ( $\sim 0.4$  h<sup>-1</sup>), moderate ( $\sim 0.2$  h<sup>-1</sup>) or low degraders ( $\sim 0.05$  h<sup>-1</sup>, based on daidzein disappearance phenotypes) (Zheng et al. 2003). Low degraders have greater apparent absorption (urinary excretion) of isoflavones whereas high degraders have lower urinary excretion. Zheng et al. (2004) found that high daidzein degraders ( $n=13$ ;  $k=0.36 \pm 0.01$  h<sup>-1</sup>) had significantly lower bioavailability of daidzein ( $43.1 \pm 5.0$  vs.  $60.6 \pm 5.1\%$  ingested dose) and glycitein ( $41.4 \pm 6.4$  vs.  $68.6 \pm 6.6\%$  ingested dose) as reflected in mean urinary excretion compare to low daidzein degraders ( $n=12$ ;  $k=0.14 \pm 0.01$  h<sup>-1</sup>). The gap in knowledge needs to be filled about what occurs between ingestion and absorption of isoflavones. *In vitro* fecal incubation with isoflavones showed disappearance of these compounds over time. Gut bacterial activity was identified as responsible for this degradation, as it did not occur with autoclaved fecal material (Hendrich et al. 1998).

Identification of the specific microbial species or strains catabolizing isoflavones must be done to fully understand isoflavone bioavailability. Several techniques have facilitated the identification of the complex composition of microbial ecology. Based on molecular biology procedures, one of these techniques, polymerase chain reaction coupled with denaturing gradient gel electrophoresis (PCR-DGGE) was reported by Muyzer et al. (1993). The principle of PCR-DGGE is to multiply the 16S rRNA gene variable region that seems to be a unique “signature” DNA sequence for each bacterial species. To obtain an optimum separation of the different 16S rRNA gene segments based on their GC content, DGGE must be performed with an appropriate urea gradient. This technique can be applied to virtually any type of microbial environment and became a useful tool to assess microbial composition of fecal samples with relevance to gut microbial ecology. Indeed, PCR-DGGE



can be applied to bacterial communities from soil and rhizosphere (Smalla et al. 2001), water (Murray et al. 1996), feces from animals (Simpson et al. 1999) or humans (Walter et al. 2001).

Our hypothesis is that humans with different fecal isoflavone degradation phenotypes have different gut microbial ecologies and that individuals sharing similar *in vitro* fecal degradation rates also possess similar microorganisms that could be involved in degrading isoflavone, as reflected by PCR-DGGE analysis. To that end, we screened fecal samples from a small but diverse human population, compared *in vitro* fecal isoflavone degradation rates with microbial DNA band patterns obtained after PCR-DGGE, sequenced bands, identified the corresponding species using a DNA sequence database, and tested the ability of these identified species to degrade isoflavones in simulated human gut/fecal environments.

## MATERIAL AND METHODS

### Human studies

This project was approved by the Institutional Review Board of Iowa State University.

In a preliminary study, one subject gave a fresh fecal sample one month apart in November and December 2003. Each sample was assessed for *in vitro* fecal isoflavone degradation and gut microbial profile analysis.

In a larger scale study, 33 subjects were recruited at Iowa State University and surrounding areas for this study. Eligibility criteria included age of 18 to 40 years, no use of antibiotics in the past three months and no bowel diseases (e.g., colitis). Users of oral contraceptives or smokers were not excluded. Subjects were asked to avoid consumption of soy-containing foods (a list of selected food items were given to subjects) three days before giving a fresh fecal sample and instructed to avoid exposure of the sample to air insofar as possible, by compressing a plastic bag around the sample and excluding air from the sample bag as soon as possible.

### Fecal Incubation

Feces in closed bags were mixed by hand to obtain more homogenous samples. One gram of feces was added to 30 mL of sterile anaerobic Brain Heart Infusion (BHI) media (Fisher, Chicago, IL) prepared with 4 g sodium bicarbonate/L media as a buffer and 20 mL cysteine sulfide (Sigma, St Louis) as an oxygen indicator. Chemically synthesized daidzein, genistein and glycitein were added to the fecal BHI mixture to obtain a final concentration of 100  $\mu$ M for each isoflavone. Isoflavones were synthesized chemically according to Chang et al. (1994) and Lang'at-Thoruwa et al. (2003). Duplicate tubes were incubated for each sample as well as a negative control (feces + BHI). Each tube was then vortexed for 10 sec to homogenize feces and isoflavones and duplicate samples taken immediately afterwards and frozen at  $-60^{\circ}\text{C}$  to measure isoflavone concentration at baseline. Tubes were placed at  $37^{\circ}\text{C}$  and isoflavone concentration over time was assessed by taking duplicate samples from each tube at 3, 6, 9, 12 and 24 h. All the samples taken were put at  $-60^{\circ}\text{C}$  until isoflavone extraction and HPLC analysis. Incubation tubes were maintained anaerobically at all times during this process to preserve bacterial quality.

### Isoflavone extraction from fecal incubation samples and HPLC analysis

Isoflavone extraction from fecal *in vitro* incubations was done using a protocol from Zheng et al. (2003). Samples were thawed and centrifuged at 13,000 g for 2 min. 1 mL of the supernatant was loaded on a pre-wetted Sep-Pak C18 cartridge (Waters, Milford, MA) along with 50  $\mu$ L of 2 mg/mL THB (2,4,4'-trihydroxydeoxybenzoin) in 80% methanol used as an internal standard.. The column was washed with 2 mL of deionized water to eliminate water-soluble contaminants. Isoflavones were then eluted with 1 mL 80% methanol, filtered using 17 mm PTFE filters (Alltech, Deerfield, IL) into an HPLC vial and stored at  $4^{\circ}\text{C}$  until analysis. HPLC analysis of the samples was done using a protocol established by Zhang et al. (1999) with 100% acetonitrile (ACN) and 0.1% glacial acetic acid (GAA) solution. Starting gradient was 15% ACN / 85% GAA, 50% / 50% at 22 min, 100% / 0% at 15 min, 15% / 85% at 28 min followed by an equilibration phase of 3 min at 15% / 85%. Degradation rates were established by calculating the natural logarithm of the percentage remaining isoflavones over time. All 4 values were considered (2 tubes/sample and 2 samples/time point) at each time

point for each subject to establish the isoflavone degradation rate. To quantify isoflavones, standard curves for each compound were established using chemically synthesized isoflavones and analyzed by reverse-phase HPLC using the same protocol as above. We focused solely on isoflavone disappearance over time in this project. Thus, isoflavone metabolites were not investigated here.

#### Bacterial DNA extraction from fecal incubation

At baseline of the fecal incubation, 2 samples from each tube for each individual were taken and immediately frozen at  $-60^{\circ}\text{C}$ . To extract DNA from fecal bacteria, a DNA extraction kit from Promega (Madison, WI) was used and its associated protocol modified for fecal bacterial DNA extraction. 500  $\mu\text{L}$  of fecal incubation mixtures were mixed with 500  $\mu\text{L}$  of 50 mM EDTA. 60  $\mu\text{L}$  of lysozyme (Sigma, St Louis, MO), 35  $\mu\text{L}$  lyzostaphin (Sigma) and 60  $\mu\text{L}$  cell lysis solution (Promega) were added to that mixture and incubated at  $37^{\circ}\text{C}$  for 1 h. Samples were then centrifuged at 14,000 g for 2 min, the supernatant discarded and the pellet redissolved in 600  $\mu\text{L}$  nuclei lysis solution (Promega). Heating for 5 min at  $80^{\circ}\text{C}$  was followed by a cool-down 15 min at room temperature and 30 min incubation with 3  $\mu\text{L}$  RNase at  $37^{\circ}\text{C}$ . 200  $\mu\text{L}$  of protein precipitation solution (Promega) were then added to the RNase treated mixture, vortexed for 20 sec and incubated on ice for 5 min. Samples were then centrifuged 3 min at 14,000 g and the supernatant transferred to a clean 1.5 mL tube containing 600  $\mu\text{L}$  of isopropanol in order to condense DNA into a visible mass. Following another 2 min centrifugation, DNA was washed with 600  $\mu\text{L}$  70% ethanol, and resuspended in TE buffer (10 mM Tris, 1 mM EDTA). DNA concentration ( $\text{ng}/\mu\text{L} = A_{260} \times 50 \times \text{dilution factor}$ ) and purity ( $= A_{260}/A_{280}$ ) were then measured using a Beckman DU® 640 spectrophotometer (Beckman, Schaumburg, IL) at 260 and 280 nm wavelengths.

#### Bacterial 16S rDNA amplification

Bacterial DNA was amplified using a touchdown PCR method, which decreases the annealing temperature over time ( $1^{\circ}\text{C}$  every other cycle) in order to target non-specifically all bacterial 16S rRNA gene variable region sequences. Primers were synthesized to target non-specifically the constant region of the variable region of the rRNA gene. Three primers were

used for that PCR: 1 forward (3'GGTCGTCGGCGCCATTA5'), 1 reverse (3'GACGACGGAGGGCATCC5') and 1 reverse with a GC clamp (3'GACGACGGAGGGCATCCGGGGGGGCACGGGGGCGGGGCGGGCGGCGCGCCGCCCGC5'). Each PCR mixture contained (final concentration): 1 µl each primer (1 pMol), 1 µl dNTP mixture (50 pMol each dNTP), 5 µl of nuclease-free PCR buffer (500 mMol KCl, 100 mMol Tris HCl pH=9 @ 25°C, 1.0% Triton X-100 and 15 mMol MgCl<sub>2</sub>), 28 µl water and target DNA (8 ng/µl PCR mixture). Primers were synthesized by Sigma Genosis (The Woodlands, TX) and PCR components obtained as a kit from Promega (Madison, WI). After the first step of the PCR program (4 min 94°C), 1 µl nuclease-free PCR buffer, 0.5 µl water and 0.5 µl Taq polymerase at 5 units/µl were added. The PCR program was established as followed: 4 min 94°C, 20 cycles with touchdown: 1 min 94°C, 1 min 65°C (decrease 1°C every other cycle), 3 min 72°C; 10 regular cycles: 1 min 94°C, 1 min 55°C, 3 min 72°C; 7 min 72°C; cool down at 4°C. PCR products should be around 200 base pair (bp) and were checked using a 0.1% agarose gel with ethidium bromide and a PCR ladder used as a standard (Sigma). A UV lamp was used to visualize DNA and compare it to a standard DNA PCR ladder (mixture of 50, 150, 300, 500, 750 and 1,000 bp DNA fragments) commercially available (Sigma, St. Louis, MO). DNA concentration was measured using a Beckman DU® 640 spectrophotometer (Beckman, Schaumburg, IL) at 260 and 280 nm wavelength.

#### Denaturing gradient gel electrophoresis

Two acrylamide stock solutions were used to prepare the gel: 100% urea denaturing solution (42 g urea, 16.9 mL acrylamide, 2 mL 50×Tris Acetate EDTA (TAE), 40 mL 100% formamide and made to 100 mL with distilled deionized (dd) water) and 0% urea denaturing solution (16.9 mL acrylamide, 2 mL 50×TAE and made to 100 mL with dd water). To obtain an optimum separation of the bacterial 16S rRNA gene sequence mixtures, 2 acrylamide gradients were used: 32.5-45% urea and 35-50% urea. The different acrylamide concentrations were done as follow: for example, 50% urea in acrylamide solution was prepared by mixing 50 mL 100% and 50 mL 0% urea denaturing solutions. DGGE was done using a D-Code universal mutation detection system from Bio-Rad (Hercules, CA).

Acrylamide gels were prepared as follows: smooth edged glass plates (front plate: 16×20 cm; back plate: 18.5×20 cm; previously cleaned with 100% ethanol). A PAG film (Cambrex Bio Science Rockland Inc. Rockland, Maine) was cut with a razor blade to fit the back plate. The hydrophilic side of the PAG film was in contact with the gel, the hydrophobic with the back plate. To assure that the film was secured to the back plate, water was put between the film and the plate and visible air bubbles completely removed from under the film. Spacers were covered with cello-seal (Fisher, Chicago, IL) and put on each side of the back plate covered with the PAG film. The front plate was placed on top of spacers and the entire apparatus was clamped according to instructions provided with the D-code system. The bottom of the sandwich was covered with a thin film of Cello-Seal and the apparatus clamped over a water-proof seal in order to avoid leakage when pouring the gel. The gel was poured with a Hoefer SG 50 gradient former (Amersham Biosciences, Piscataway, NJ) with a stir bar in each chamber on medium speed. The gel was poured within 3 min or less and a 16 well comb inserted at the top of the gel. Polymerization of the gel occurred within 30 min. Samples were normalized by loading in each well 2000 ng DNA PCR product samples previously mixed with 6  $\mu$ L DGGE loading dye. The gel was then run at 160 V for 3.5 h, and cooled down by turning off and opening the top part of the D-code system for 20 min before removing the sandwich apparatus. The gel was silver stained by washing 3 x 1 min with dd water, adding 200 mL solution I (200 mg  $\text{AgNO}_3$  in 200 ml  $\text{H}_2\text{O}$ ) for 15 min, washing 2 x 1 min with dd water, adding 200 ml of solution II (20 mg  $\text{NaBH}_4$ , 0.8 ml 37% formaldehyde, 3 g NaOH in 200 ml) for 20 minutes and washing 4 x 1 min with dd water (Liu and Ritalahti, 1996). The gel was then scanned using a GS-800 calibrated imaging densitometer (Bio-Rad, Hercules, CA) and an image obtained for analysis of DNA bands using Quantity One software (band migration and intensity and lane profiles). After analysis, the bands of interest were excised and DNA eluted following a protocol from Rieman and Winding (2001). Using a sterile razor blade, pieces of acrylamide containing the DNA of interest were put in 40  $\mu$ L of 1×SSC buffer in a 0.6 mL sterile tube. Samples were incubated overnight at room temperature and 2 h at 37°C the next morning, then centrifuged to precipitate acrylamide pieces, the supernatant containing the DNA transferred into new sterile tubes containing 3 volumes of 96% ethanol and 0.1 volume of 8 mol/L lithium

chloride. DNA was then precipitated by centrifuging the tubes 3 min at 14,000 g, discarding the supernatant, and dissolving the DNA in 15  $\mu$ L TE (10 mM Tris, 1 mM EDTA) Buffer.

#### DNA amplification from acrylamide gels, sequencing and identification of the micro-organisms from 16S rDNA sequence

DNA eluted from acrylamide was re-amplified using the same protocol as described earlier, with a slightly different PCR mixture (15  $\mu$ L TE buffer containing the target DNA eluted as above, 1  $\mu$ L each primer (5 pMol), 1  $\mu$ L dNTPs mixture (20 pMol each dNTP), 5  $\mu$ L buffer with 15 mMol MgCl and 24  $\mu$ L dd water. After 4 min 94°C, the TAQ mixture was added as described before. After PCR, the samples were checked for length of the products (200 bp, using a standard DNA ladder as above) using a 0.1% agarose gel, purified using a PCR purification kit from Promega (Madison, WI) and quantified as described before. For sequencing, PCR samples concentrations were 50  $\mu$ g/ $\mu$ L and the primer with the lowest T<sub>m</sub> or % GC was at 10 pmol/L. Sequencing was performed by the Iowa State University DNA sequencing facility and results checked to confirm sequence identity. The final sequence was matched to a library of 16S rRNA gene sequences using Basic Local Alignment Search Tool (available at <http://www.ncbi.nlm.nih.gov/BLAST/>). 100 possible matches were provided for each sequence and the possible matches that corresponded to known microorganisms were considered as possible species that degraded isoflavones (Overall PCR/DGGE protocol summarized, Fig 1).

#### Culture of selected micro-organisms

Putative isoflavone degrading microorganisms selected from the BLAST analysis were purchased as pure single strains from the American Type Culture Collection (Manassas, VA): *Bacteroides eggerthii* (ATCC 27754), *Bacteroides uniformis* (ATCC 8492), *Bacteroides vulgatus* (ATCC 31376), *Bacteroides fragilis* (ATCC 43858), *Bacteroides ovatus* (ATCC 700292), *Bacteroides thetaiotaomicron* (ATCC 29148), *Prevotella ruminicola* (ATCC 19189), *Prevotella oralis* (ATCC 33322), *Prevotella pallens* (ATCC 700821), *Prevotella veroralis* (ATCC 33779), *Tannerella forsythensis* (ATCC 43037), *Ruminococcus obeum* (ATCC 29174), *Clostridium orbiscindens* (ATCC 49531), *Eubacterium ramulus*

(ATCC 29099), *Fusobacterium prausnitzii* (ATCC 27766) and *Porphyromonas gingivalis* (ATCC 33277). *Bacteroides acidifaciens* (DSM 15896) was purchased from the German Collection of Microorganisms and Cell Culture (Braunschweig, Germany). To revive the freeze-dried culture, a specific medium was prepared following protocols provided by ATCC. The medium contained ( $L^{-1}$ ): 50 g Brain heart infusion powder, 30 g peptone, 25 mL 1 N sodium hydroxide, 5 g yeast extract, 5 g glucose, 1 g starch, 1 g cellobiose, 1 g maltose, 10 mL hemin solution (0.5 g hemin/L dd water), 200  $\mu$ L vitamin K1 solution (0.5 mL vitamin K1 solution/L 95% ethanol), 20 mL cysteine sulfide (prepared with 10 g cystein HCl and 10 g sodium sulfide per L dd water, pH=10.0), 1 mL 0.1% resazurin and 100 mL/L of sterile rumen fluid (obtained from Dr. Mark Rasmussen, National Animal Disease Center, Ames, IA). The medium was anaerobic and flushed with 80%  $N_2$  – 20%  $CO_2$ , then tightly closed and sterilized in 20 mL aliquots. Freeze-dried microbial cultures were anaerobically and aseptically transferred into the sterile medium and placed in an incubator-shaker for 24-48 h at 37°C and 140 rpm. Once grown, the bacteria were transferred into sterile 1.5 mL centrifuge tubes, centrifuged at 14,000 g for 2 min to pellet the cells, the supernatant removed and the bacteria frozen at -4° then -60°C until further growth and use required.

#### Fecal incubation with selected micro-organisms in nutrient-rich or nutrient-poor media

To perform fecal incubation with or without selected microorganisms, we selected 2 different media: nutrient rich and nutrient poor. The nutrient rich media was the same as described for revival of microorganisms. To create a nutrient poor environment, BHI media (similar to the one used for screening fecal isoflavone degradation) was deprived of nutrients by incubating 1 g fresh human fecal sample from one subject overnight. After this overnight incubation, 2.5 mL of overnight grown culture of selected microorganisms were added, either alone or in combination, and the bacteria allowed settling for 6h before adding isoflavones. In vitro degradation was then assessed as above. In nutrient-rich media, 1 g of fresh fecal sample with the selected microorganism(s) (2.5 mL of overnight grown culture) were added to 30 mL media and allowed to settle for 6h, before adding isoflavones.

Two separate sets of incubations using nutrient rich and poor media were performed. Between both sets, all species identified by DGGE were screened for isoflavone degradation. Fecal samples from three individuals were considered for each set.

The first set of microbial culture isoflavone degradation studies included fecal samples from a Caucasian male (26 years of age), an Asian female (24 years) and an Indian male (18 years), all previously screened as moderate or moderate/low daidzein degraders during screening of the original 33 subjects. Treatments included (duplicate tubes for each treatment and each fecal sample): *P. oralis*, *P. pallens*, *P. ruminicola*, *B. eggerthii*, *B. fragilis*, a mixture of *Prevotella* (*ruminicola*, *oralis*, *pallens* and *veroralis*), a mixture of *Bacteroides* (*eggerthii*, *thetaitaomicron*, *fragilis* and *vulgatus*) and a mixture of Bacteroidales (all 4 *Prevotella* and *Bacteroides* together + *Porphyromonas gingivalis*). Despite not finding significant microorganisms of interest related to daidzein degradation, we still included that compound in all incubations along with genistein and glycitein.

The second set of microbial culture isoflavone degradation studies included fecal samples from a Caucasian male (same subject as in the first set of incubations), an African-American female (26 years of age) and an Asian female (30 years), all previously screened as moderate or moderate/low daidzein degraders during screening of the original 33 subjects. Treatment tubes included re-evaluation of *B. fragilis* and *P. ruminicola* in nutrient rich and poor-media. Strains identified by DGGE but not included in the first set of incubations were investigated in this second set. Species investigated in nutrient-poor vs. nutrient rich media were *Bacteroides ovatus*, *Bacteroides acidifaciens*, *Porphyromonas gingivalis*, *Ruminococcus obeum*, *Eubacterium ramulus*, *Clostridium orbiscindens*, *Fusobacterium prausnitzii* and *Tannerella forsythensis*. Due to limitation in time and supplies, we investigated in nutrient-poor only *Prevotella veroralis*, *Bacteroides thetaitaomicron*, *Bacteroides uniformis* and *Bacteroides vulgatus*. Species selected for these incubations were selected from DGGE and literature data only. Microorganisms not expected to degrade isoflavone were not included in this project.

All isoflavone degradation rate results were expressed as a percentage of control (feces and isoflavones only) response for a final concentration of  $10^9$  cells added/mL media. Cell concentration after overnight growth varied from  $0.6 \times 10^9$  cells/mL media for *T.*



*forsythensis* to  $1.2 \times 10^9$  cells/mL media for *Bacteroides vulgatus*. Normalization expressing results as a percentage was done because control degradation rate differed between individuals and isoflavones; normalization of cell concentration allowed comparison among treatments.

### Statistical analysis

All statistical analyses were performed using SAS (SAS Institute, version 6.12; 1998, Cary, NC). One-way ANOVA and multiple comparisons were used to compare band intensity, degradation rates between clustered isoflavone degrader phenotypes and among incubation with bacterial species in nutrient rich and poor incubations.

## **RESULTS**

### Interindividual short-term difference in isoflavone degradation rate and gut microbial profile

Comparison of degradation rates established in November and December 2003 for the same subject (Caucasian male, age 24) showed that daidzein ( $0.0458$  vs.  $0.0421 \text{ h}^{-1}$ ) and genistein ( $0.127$  vs.  $0.107 \text{ h}^{-1}$ ) degradation rates were similar at both times. However, a ~2.5 fold decrease in glycitein degradation rate was observed between November ( $0.1988 \text{ h}^{-1}$ ) and December ( $0.5385 \text{ h}^{-1}$ ). DGGE analysis of gut microbial profile at both time points (results shown in duplicate for each time point) showed 5 bands (Band A through E) of greater intensity (Table 1) in the fecal sample taken in November compared to that taken in December (Figure 2), while bands F and G were in greater intensity in December compared to November.

### In vitro screening of fecal isoflavone degradation rates

Thirty-three subjects were selected for this screening (20 men, 13 women; mean age was  $25.6 \pm 4.4$  years; 45.5% Caucasian, 24.2% Indian, 18.2% Asian and 12.1% African American). All subjects were screened for their fecal isoflavone degradation over 12 h. Segmented analysis over time revealed that most individuals differed in fecal isoflavone degradation between the first 6 h and the second 6 h of incubation (increase, decrease or

stable). There was no correlation between 0-6 h and 6-12 h rates for daidzein ( $r=0.11$ ;  $p=0.55$ ), glycitein ( $r=0.26$ ;  $p=0.31$ ), and genistein ( $r=0.17$ ;  $p=0.61$ ). Figure 3 described the segmented degradation rates between 0-6 h and 6-12h for genistein when still quantifiable by time 12 h (data not shown for daidzein and glycitein). With respect to these changes over time, a DGGE of bacterial 16S rDNA of time 0 and 12 h incubation of one individual was run to assess stability of the fecal flora *in vitro* over time (Figure 4). Analysis of the gel showed several changes in band patterns (increased or decreased band intensity), suggesting significant changes over time when assessing the human fecal flora *in vitro*. Considering the differences in segmented degradation rates and gut flora observed in figures 3 and 4, selection and ranking of individuals were done based on their 0-6h degradation rates, assuming a more accurate reflection of the gut microbial metabolism and physiology. 0-6 h isoflavone degradation rates were significantly albeit weakly correlated with one another (daidzein vs. glycitein:  $r=0.1343$ ,  $p=0.035$ ; daidzein vs. genistein:  $r=0.2059$ ,  $p=0.008$ ; glycitein vs. genistein:  $r=0.2656$ ,  $p=0.002$ ). However, the highest and lowest degradation rates of all three isoflavones did not correspond completely to the same individuals. Therefore, we investigated the fecal microbial DNA profiles of high and low degraders for each isoflavone separately.

#### DGGE of fecal microbial DNA in high vs. low isoflavone degraders

The four highest and lowest degraders for each isoflavone were selected for DGGE analysis. Subjects 11, 21, 29, 28 and 18, 20, 4, 1 were low and high daidzein degraders, respectively (daidzein degradation rates of  $0.051 \pm 0.032$  vs.  $0.304 \pm 0.096 \text{ h}^{-1}$ ;  $p=0.002$ ). Subjects 21, 33, 9, 2 and 18, 26, 13, 4 were low and high glycitein degraders, respectively ( $0.084 \pm 0.025$  vs.  $0.574 \pm 0.299 \text{ h}^{-1}$ ;  $p=0.017$ ). Subjects 11, 28, 33, 2 and 4, 5, 18, 19 were low and high genistein degraders, respectively ( $0.146 \pm 0.034$  vs.  $1.47 \pm 0.14 \text{ h}^{-1}$ ;  $p<0.0001$ ).

DGGE analysis of fecal microbial profiles associated with glycitein degradation showed 5 bands of greater intensity in high compared to low degraders (Gly 1 to Gly 5) (Figure 5). Comparison of gut microbial profiles associated with genistein degradation also showed 5 bands of greater intensity present in high genistein degraders (Gen 1 to Gen 5)

(Figure 6). However, daidzein high degraders did not show any significant bands of greater intensity compared to low daidzein degraders.

#### Sequencing and identification of micro-organisms from the bands of interest

Sequencing and matching of the sequences using BLAST gave for each band 100 possible matches to known and unknown bacteria (Figures 2, 5 and 6). The overall results from all bands associated with isoflavone degradation gave strong similarities in microorganism identification. Indeed, all known microorganisms possibly related to bands present in high degraders were from the CFB group (Cytophagia-Flavobacteria-Bacteroides) and *Clostridium* genus. Most of the possible matches corresponded to *Bacteroides*, *Prevotella*, *Clostridium*, *Ruminococcus* and *Fusobacterium*. Based on BLAST analysis, we selected the following strains: *Bacteroides fragilis* (band A), *Bacteroides uniformis* (Band Gen 3), *Bacteroides ovatus* (Band G), *Bacteroides vulgatus* (Band C), *Bacteroides thetaiotaomicron* (Band Gen 3), *Bacteroides eggerthii* (Band gly 2), *Porphyromonas gingivalis* (Band B), *Prevotella pallens* (Band E), *Prevotella ruminicola* (Band D), *Prevotella oralis* (Band F), *Prevotella veroralis* (Band F), *Ruminococcus obeum* (Band Gly 4), *Fusobacterium prausnitzii* (Band Gen 5), *Bacteroides acidifaciens* (Band Gly 3), *Tannerella Forsythensis* (Band Gly 1). We also selected the following strains from published literature describing isoflavone degradation: *Eubacterium ramulus* (Schoefer et al. 2002) and *Clostridium orbiscindens* (Schoefer et al. 2003). All DGGE bands had significant differences in mean band intensity between duplicate bands at each time point one month apart when establishing intra-individual variability of degradation (Table 1). Significance was also observed when comparing mean band intensity of low (n=4) and high (n=4) glycitein and genistein degraders (Table 1). These strains were first investigated in several different preliminary experiments with small power then re-investigated using a higher power.

#### Preliminary findings

Single strain incubation with basic BHI media did not show any significant isoflavone degradation over 24 h incubation for *E. ramulus*, *B. ovatus*, *B. fragilis*, *B. vulgatus* and *P. veroralis* (data not shown). Other strains were not tested for this preliminary screening.

Incubation with autoclaved fecal suspension in BHI media and subsequent added microorganisms also failed to show any significant isoflavone degradation (data not showed).

Fecal incubation with fresh fecal suspension in basic BHI media and added microorganisms showed similar 0-9 h degradation rates compared to control. Strains tested here were *B. fragilis*, *B. ovatus*, *B. thetataomicron*, *B. vulgatus*, *B. uniformis*, *P. veroralis*, *E. ramulus* and *C. orbiscindens*. Only one subject was screened for this preliminary experiment. A substantial decrease (~2-fold;  $p < 0.05$ , based on duplicate samples for each incubation treatment) in 6-9 h degradation rate was observed in the control (feces and isoflavone only) while bacterial strains added to the fecal suspension maintained *in vitro* isoflavone degradation rate constant and higher than control during that 3h period (Figure 7). *B. thetataomicron* and *B. uniformis* showed a significantly higher rate of degradation between 6-9 h compared to the overall 0-9 h rate ( $p < 0.05$ ). Considering that the presence of selected microorganisms in a fecal suspension had a greater influence on isoflavone disappearance compare to control when nutrients were less available, we screened and compared bacterial metabolism of different strains in two separate media, one poor and one rich in nutrients.

#### First set of incubation in nutrient-rich and –poor media

Analysis of baseline isoflavone degradation rates in control incubations (feces + isoflavone) showed that mean daidzein and genistein rates were similar between nutrient-rich and nutrient-poor media ( $0.051 \pm 0.04$  vs.  $0.040 \pm 0.012 \text{ h}^{-1}$  and  $0.074 \pm 0.027$  vs.  $0.052 \pm 0.028 \text{ h}^{-1}$  in nutrient rich vs. nutrient poor for daidzein and genistein, respectively;  $p > 0.05$ ). However, glycitein showed a ~4-fold mean increase of degradation rate in nutrient-poor compared to nutrient-rich media ( $0.153 \pm 0.064$  vs.  $0.026 \pm 0.012 \text{ h}^{-1}$ , respectively;  $p < 0.05$ ) (Figure 8).

In nutrient poor media, we observed an increase in mean degradation rate for all three isoflavones for each bacterial species tested. These increases did not correlate with initial control degradation rates of each individual (data not shown). However, due to large standard deviations, only a few results showed significant differences. Combinations of bacteria failed to show a significant increase compared to control in nutrient poor media. Daidzein disappearance rate was significantly increased ( $p < 0.05$ ) by the presence of *P. pallens* ( $119 \pm$

30% increase over control response) and *P. oralis* ( $287 \pm 105\%$  increase over control response). Glycitein disappearance rate was significantly increased by the presence of *P. oralis* ( $193 \pm 54\%$  increase over control response) and *B. eggerthii* ( $146 \pm 29\%$  increase over control response) and genistein by the presence of *P. pallens* ( $165 \pm 74\%$  increase over control response), *P. oralis* ( $263 \pm 74\%$  increase over control response), *B. eggerthii* ( $196 \pm 92\%$  increase over control response) and *B. fragilis* ( $156 \pm 56\%$  increase over control response) (Figure 9).

In nutrient rich media, much smaller increases in degradation rates compared to control were observed. These increases did not correlate with initial control degradation rates of each individual (data not shown). Statistical significance was obtained for daidzein disappearance by the presence of Prevotella (mixture of *P. ruminicola*, *oralis*, *pallens* and *veroralis*) ( $23 \pm 9\%$  increase over control response) and genistein disappearance by the presence of *P. pallens* ( $33 \pm 10\%$  increase over control response). Glycitein rates showed decreases in percentage of control response with significance when *B. eggerthii* ( $-56 \pm 8\%$  decrease over control response) was added to the fecal suspension (Figure 10).

#### Second set of incubation in nutrient-rich and –poor media

With two new subjects and one recalled from the first set of incubations, analysis of baseline isoflavone degradation rates in control (feces + isoflavone) showed a marginally significant increase of daidzein degradation rate in nutrient rich compared to nutrient poor media ( $0.031 \pm 0.006$  vs.  $0.098 \pm 0.061 \text{ h}^{-1}$ ). Glycitein degradation rates were similar in both media ( $0.219 \pm 0.141$  vs.  $0.171 \pm 0.092 \text{ h}^{-1}$  in nutrient poor and rich, respectively) while mean genistein degradation rate significantly increased in nutrient-rich media ( $0.223 \pm 0.112$  vs.  $0.049 \pm 0.016 \text{ h}^{-1}$  in nutrient rich and poor, respectively) (Figure 8). The same subject investigated in both sets of incubations had degradation rates that followed the overall results obtained with all 3 subjects for each set of incubations.

Data obtained from nutrient poor incubations produced a greater range of mean percentage increase in isoflavone degradation rates compared to the first set of incubations (Figure 9). These increases did not correlate with initial control degradation rates of each individual (data not shown). Despite observing increases in percentage degradation rates

from the control, some strains produced very little effect while other gave a mean percentage increase up to more than 800% when added to the fecal suspension. For example, the presence of *B. thetaiotaomicron* gave only  $46 \pm 32\%$  increase over control response in genistein degradation rate while daidzein and glycitein increased by  $10 \pm 25$  and  $7 \pm 27\%$  increase over control response. On the other hand, adding *B. acidifaciens* to a nutrient-poor media produced  $789 \pm 249$ ,  $584 \pm 222$  and  $753 \pm 63\%$  increases over control response of daidzein, glycitein and genistein degradation respectively, all with  $p < 0.05$ . Trends similar to *B. acidifaciens* were observed with *B. uniformis* and *T. forsythensis*. The latter did not show a significant increase in glycitein degradation over control due to a large standard deviation ( $469 \pm 320\%$  increase over control response). Several other strains did not produced increases as large as *B. acidifaciens*, but due to a similar increase of isoflavone degradation rates among fecal samples of all three subjects (small standard deviation), the percentage increase compared to the control response was significant ( $p < 0.05$ ). Daidzein degradation was significantly increased by the presence of *P. ruminicola* ( $206 \pm 108\%$  increase over control response), *B. vulgatus* ( $104 \pm 40\%$  increase over control response), *E. ramulus* ( $210 \pm 55\%$  increase over control response), *C. orbiscindens* ( $469 \pm 95\%$  increase over control response) and *F. prausnitzii* ( $94 \pm 40\%$  increase over control response). Glycitein degradation was significantly affected by the presence of *P. veroralis* ( $117 \pm 59\%$  increase over control response), *B. ovatus* ( $106 \pm 17\%$  increase over control response) and *C. orbiscindens* ( $181 \pm 71\%$  increase over control response) whereas strains increasing genistein degradation were *B. ovatus* ( $106 \pm 17\%$  increase over control response) and *E. ramulus* ( $117 \pm 52\%$  increase over control response).

In nutrient rich media, several strains gave similar results to those obtained during the first set of incubations (Figure 10). These variations in percentage of control response did not correlate with initial control degradation rates of each individual (data not shown). Some decreases in percentage of the control response were observed, but not statistically significantly different from the control. The presence of *P. pallens* with respect to genistein disappearance caused a significant increase in degradation rate compared to control in a nutrient rich media during the first set of incubation ( $33 \pm 10\%$  increase over control response;  $p < 0.05$ ). In the second set of incubations (Figure 15), five strains caused significant

increases in degradation rate compare to control. Daidzein degradation was significantly affected by adding to nutrient rich media *B. acidifaciens* ( $429 \pm 95\%$  increase over control response) and *C. orbiscindens* ( $147 \pm 28\%$  increase over control response), while glycitein degradation was significantly higher in the presence of *B. ovatus* ( $78 \pm 42\%$  increase over control response), *B. acidifaciens* ( $632 \pm 35\%$  increase over control response), *E. ramulus* ( $49 \pm 19\%$  increase over control response) and *T. forsythensis* ( $251 \pm 103\%$  increase over control response). As for genistein, a statistically significant increase in degradation compared to control was observed with the presence of *B. ovatus* ( $57 \pm 29\%$  increase over control response), *B. acidifaciens* ( $615 \pm 82\%$  increase over control response), *E. ramulus* ( $33 \pm 6\%$  increase over control response), *C. orbiscindens* ( $68 \pm 25\%$  increase over control response) and *T. forsythensis* ( $236 \pm 76\%$  increase over control response).

Comparison of percentage variations over control response for *B. fragilis* and *P. ruminicola* that were investigated in both sets of incubations did not give any significant differences. Comparison of daidzein, genistein and glycitein mean percentage variation between set 1 and 2 in either nutrient rich or poor gave p values of 0.08 or above (Figures 9 and 10). Compare to control response, only *P. ruminicola* showed a significant increase of daidzein degradation in the second set of experiment ( $206 \pm 108\%$  increase over control response;  $p < 0.05$ ).

## DISCUSSION

This study was the first to investigate fecal microbial disappearance of soy isoflavones. This project not only would provide new screening tools for human isoflavone bioavailability based on gut microbial profile, but it also lays a foundation for dietary interventions to decrease isoflavone-degrading human gut microorganisms in order to improve absorption and health-promoting effects of isoflavones. We hypothesized that feces of high isoflavone degraders share isoflavone degrading bacterial strains not seen in feces of low isoflavone degraders. Developed by Muyzer et al. (1993), PCR-DGGE is a molecular tool to characterize microbial populations. This technique was based on targeting and multiplying by PCR the DNA sequences coding for the V3 region of 16S rRNA with primers

designed to match the conserved region of the 16S rRNA gene. This sequence is different from one species to another and highly conserved within the same species. The 16S rDNA V3 region sequence is a unique “signature” of a microbial species. Theoretically, PCR of the 16S rRNA sequences of a bacterial community should represent the original population, both in a quantitative and a qualitative manner. The rationale for using PCR-DGGE techniques is because of their advantages in studying complex microbial ecology: easiness, reproducibility, reliability and speed (Muyzer and Smalla. 1998; Muyzer et al. 1993). All gels in this study were done twice from separate DNA extraction samples and separate PCRs and produced the same results. By comparing standard microbiological techniques with PCR-DGGE, it has been shown that touchdown PCR gives an accurate estimate of the original bacterial population present in the sample as reflected in bacterial 16S rDNA. The intensity of the bands obtained after staining should represent the frequency of the species in the original sample studied (Murray et al. 1996). The use of touchdown PCR with a decreasing annealing temperature from 65 to 55°C enables the PCR primers to target all 16S DNA sequences that vary from one another in their GC content, thus in their melting and annealing temperatures. Silver staining was used to reveal the DNA band profiles on the gel but one of the disadvantages of using this technique is that only species represented at  $\geq 1\%$  of the total bacterial population will be visualized (Muyzer et al. 1993). Thus, minor species cannot be studied using this technique and gut microbial populations are still only partially understood (Muyzer et al. 1993; Fuller and Perdígón. 2003).

With respect to isoflavone fecal in vitro degradation rates in the present study, selection of the 4 highest degraders gave respective degradation rates for daidzein, glycitein and genistein of  $0.304 \pm 0.096 \text{ h}^{-1}$ ,  $0.574 \pm 0.299 \text{ h}^{-1}$  and  $1.47 \pm 0.14 \text{ h}^{-1}$  while those of low degraders were  $0.051 \pm 0.032 \text{ h}^{-1}$ ,  $0.084 \pm 0.025 \text{ h}^{-1}$  and  $0.146 \pm 0.034 \text{ h}^{-1}$ . Focusing only on daidzein and genistein, Hendrich et al. (1998) found degradation of high daidzein and genistein degraders (n=5) to be  $0.299$  and  $0.299 \text{ h}^{-1}$ , respectively. Low degraders (n=5) had rates of  $0.012$  and  $0.023 \text{ h}^{-1}$  for daidzein and genistein, respectively. Zheng et al. (2003) found that mean high (n=13) vs. low (n=12) daidzein degradation rates were  $0.44 \pm 0.02 \text{ h}^{-1}$  and  $0.06 \pm 0.02 \text{ h}^{-1}$ , respectively in Asian women. Similar mean rates were found for Caucasian women who were high (n=4;  $k=0.47 \pm 0.03 \text{ h}^{-1}$ ) and low (n=13;  $k=0.08 \pm 0.02 \text{ h}^{-1}$ )



daidzein degraders. Mean high ( $n=25$ ;  $k=0.47 \pm 0.02 \text{ h}^{-1}$ ) and low ( $n=10$ ;  $k=0.19 \pm 0.04 \text{ h}^{-1}$ ) genistein degradation rates in Asian women were similar to those of Caucasian women who were high ( $n=18$ ;  $k=0.45 \pm 0.03 \text{ h}^{-1}$ ) and low ( $n=15$ ;  $k=0.18 \pm 0.02 \text{ h}^{-1}$ ) genistein degraders. Zheng et al. (2004) found that mean high ( $n=13$ ) vs. low ( $n=12$ ) daidzein degradation rates were  $0.36 \pm 0.01 \text{ h}^{-1}$  and  $0.14 \pm 0.01 \text{ h}^{-1}$ , respectively.

Daidzein rates were in agreement between Hendrich et al. (1998), Zheng et al. (2003) and Zheng et al. (2004) studies and the present one, but the genistein rates were much higher here. A possible explanation for that phenomenon is that we established our degradation rates on 0-6 h data, while Hendrich et al. (1998) established theirs on 0-24 h data. We decided to restrict the number of time points when establishing degradation rates because of the changes occurring in rates past 6 h (Figure 3) along with changes occurring in microbial profile when incubated over 24 h (Figure 4). To produce data as physiologically relevant as possible, we decided that 0-6 h rates and time 0 h gut microbial profiles would be more physiologically and metabolically relevant to human nutritional circumstances. These findings also question the extrapolation of *in vitro* fecal degradation rate data obtained over 24 h to *in vivo* situations. When dealing with a matrix as complex as a fecal suspension, it is essential that the data obtained are relevant to the original composition of the sample along with avoiding as much as possible any artifacts that would influence the results (Simpson et al. 1999; Moore and Moore. 1995; Fuller and Perdigón. 2003).

Comparison of microbial profile after analysis of DGGE showed how complex and different they were among individuals. Despite finding bands that were shared by several or sometimes all individuals, the overall band patterns differed from one individual to another (Figures 5 and 6) and confirms previous findings of great interindividual variability in gut microbial composition (Fuller and Perdigón. 2003). On the other hand, the presence of several bands of greater intensity found in feces of high genistein and glycitein degraders, bands that were much weaker or absent in feces of low degraders, supported our initial hypothesis that high degraders shared bands of greater intensity (Figures 5 and 6; table 1). The fact that no significant bands were found to differ between high and low daidzein degraders may be partly explained by the fact that daidzein rates were lower than those of glycitein and genistein and that only a 5-6-fold difference was observed between mean

daidzein low vs. high degradation rates while those of glycitein and genistein were 7- and 10-fold difference, respectively. Preliminary experiment done with one subject also supported our hypothesis that high isoflavone degradation was associated with DGGE bands of greater intensity (Figure 2), at least with respect to glycitein degradation, even though an n of 1 was insufficient to draw definite conclusions for daidzein or genistein as well as for extrapolation to the general population. On the other hand, dealing with the same individual gave much closer gut microbial profiles at both time points (November and December 2003) so that differences in band patterns possibly related to glycitein degradation were much easier to observe (Figure 2). Bacterial identification of the bands of interest from one individual over time (Figure 2) or comparison of 4 low versus 4 high degraders (Figure 5 and 6) gave results corresponding to the same bacterial families (*Bacteroides*, *Prevotella* and Clostridiales) led us to believe that there may be different ways to approach our initial objective of identifying isoflavone-degrading microorganisms and still obtained similar conclusions.

From microbial DNA database analysis, we selected for each DGGE band the match that gave the greatest number of hits to the DNA sequence extracted and amplified after DGGE. As a first approximation of human gut microorganisms that degraded isoflavones, this chosen sequence match corresponded to known and culturable microorganisms known to be present in the human gut (Tables 1). Despite these restrictions in the process of sequencing and identifying microorganisms, most of the DGGE bands associated with high fecal isoflavone degradation corresponded to specific microbial strain. Some bands gave 2 matches; the 2 strains selected were from the same family and very close phylogenetically. The overall results from three gels showed that higher isoflavone degradation may be related to higher levels of *Bacteroides*, *Prevotella* and certain strains of Clostridiales (Tables 1). Bands F and G obtained from one individual (figure 2 and table 1) were higher in intensity in microbial DNA from a fecal sample that showed less glycitein degradation compared with a fecal sample taken one month earlier from that subject. These bands may indicate microbial species that inhibited glycitein degradation. But the species encoded by these DNA sequences were *Bacteroides* and *Prevotella* (species also associated with higher isoflavone degradation rates), so more subtle analysis will be needed to clarify the microbial interrelationships associated with lower isoflavone degradation rates. We focused on the

hypothesis that microbial species present more predominantly in feces of high degraders would be likely responsible for isoflavone degradation. Bands A through E were thought to be responsible for glycitein degradation at a time of high degradation rate (November sample) whereas bands F and G might have corresponded to species responsible for glycitein degradation in December, where glycitein degradation was still occurring, but at a lower rate. The microbial strains tentatively identified as isoflavone degrading are some of the major human gut microbial species as *Prevotella* and *Bacteroides* can account for up to 95% of the total gut bacterial population in humans (Fuller and Perdígón. 2003).

In addition to the strains selected from DGGE, we also screened isoflavone degradation for 2 strains previously shown to degrade isoflavone or flavonoids. Schoefer et al. (2002) found that a pure strain of *Eubacterium ramulus* degraded 0.5 mM genistein completely in 4 h into 6'-hydroxy-ODMA and 2-(4-hydroxyphenyl)-propionic acid. Daidzein was degraded over 12 h by *E. ramulus* to yield ODMA at an amount equal to 70% of the daidzein initially present. In the present study, we solely focused on isoflavone disappearance over time when fecal incubations are spiked with this microorganism. Identification and quantification of metabolites will deserve further investigations. *Clostridium orbiscindens* was also selected for its capability of degrading the flavonoid quercetin (Schoeffer et al. 2003). Hur et al. (2002) also found that a *Clostridium sp.* isolated from human fecal samples (200 different unknown bacterial colonies cultured from fecal sample and each incubated with daidzein) that was capable of transforming daidzein (400  $\mu$ M) into ODMA (112  $\mu$ M) over a 3 d incubation, but we could not include this species because we could not obtain it.

Preliminary experiments done with the selected strains gave disappointing results considering that single strain incubations did not give any significant isoflavone degradation (data not shown). The same result was obtained with an autoclaved fecal matrix. Therefore, we hypothesized that if these strains were involved in degrading isoflavone, a viable fecal background would be necessary to exert a significant effect. Fresh fecal suspension with added overnight grown bacterial cultures of *Bacteroides*, *Prevotella* or *Clostridiales* (Figure 7), but with no adaptation time for the species to accommodate to the fecal environment showed that treatment degradation rates seemed to be maintained more stably and higher than in fresh fecal suspensions not containing the putative isoflavone-degrading microbial

species. This was more obvious in the last segment of the incubation period (6-9h) (Figure 7). With longer adaptation time, the presence of selected strains were better able to maintain isoflavones degradation to a higher level, even though an n of 1 done for this preliminary experiment did not allow us to draw any definite conclusions. After 6-9h incubation in brain heart infusion media, nutrient availability to the microbial species may start to decrease. This hypothesis of nutrient availability and bacterial activity of putative strains with respect to isoflavone disappearance was investigated using an *in vitro* system. Isoflavone degradation rates by bacteria added to a fecal sample were compared between two environments: one in which nutrient availability was low (nutrient-poor media) and one in which nutrient availability was high (nutrient-rich media). A rationale for this comparison came from the study of Zheng et al. (2003) who compared *in vitro* disappearance of genistein and *in vivo* genistein urinary excretion and gut transit time in 35 Asian and 33 Caucasian females. Asians with low *in vitro* fecal genistein degradation had a significantly shorter mean gut transit time and greater genistein urinary excretion compared to Asians with rapid *in vitro* fecal genistein degradation (GTT was  $40 \pm 8$ h vs.  $63 \pm 5$ h and genistein urinary excretion was  $11.0 \pm 2.7\%$  vs.  $4.0 \pm 1.7\%$  of ingested dose from soymilk for low and high genistein degraders, respectively). This was not observed in Caucasian subjects, probably because Caucasian low genistein degraders had a mean gut transit time significantly higher than Asian subjects with the same phenotype ( $86 \pm 10$ h vs.  $40 \pm 8$ h, respectively). Zheng et al. (2003) concluded that rapid gut transit time coupled with low *in vitro* genistein disappearance resulted in greater bioavailability as reflected in urinary excretion. Based on these findings, we attempted to reproduce *in vitro* a similar situation to short and long gut transit time. We hypothesized that a long gut transit time would be associated with greater nutrient fermentation in the large intestine. This would ultimately correspond to an *in vitro* nutrient-poor media in which, according to Zheng et al. (2003), greater isoflavone degradation rates would be expected. In the present study, we would expect greater isoflavone degradation rates when strains of interest are added to a fecal matrix in nutrient-poor media. The use of a nutrient-rich media would mimic an *in vivo* rapid gut transit time, where less fermentation is occurring in the colon because gut contents have less time in residence there. Thus, a nutrient-rich media would produce lower isoflavone degradation rates and much smaller effects when strains of

interests are added to the fecal suspension. An adaptation time of 6h was necessary for the bacterial species added to the fecal matrix to adapt to this environment (figure 7). Even though a standard fecal *in vitro* isoflavone rate was better assessed between 0 and 6h for correlation to DGGE (figure 3), the main goal of this part of the project was to alter significantly the original fecal flora by adding bacterial species grown separately. Modifying the *in vitro* fecal environment was not related anymore to the *in vivo* colonic environment of the subjects providing the samples. Therefore, in this particular case, adding an adaptation time should not be of concern since we focused on measuring how isoflavone disappearance is affected over time by a greater presence of the bacterial species selected.

To test this hypothesis, we designed a first set of incubation with both media and fecal samples from three individuals. We selected fecal samples from individual of both sexes and from different ethnicities. For this first set of incubations, we investigated 4 strains (*P. oralis*, *P. ruminicola*, *B. eggerthii* and *B. fragilis*) as well as a mixture of *Prevotella*, *Bacteroides* and Bacteroidales (Figures 9 and 10). All three isoflavones were investigated despite the fact that no specific microorganism was identified as a possible daidzein degrader. However, considering a similar pattern of bacteria identified for glycitein and genistein, we hypothesized that species from *Prevotella*, *Bacteroides* and Clostridiales might also have a significant effect on daidzein. To normalize and allow comparisons among treatments, we expressed the results as a percentage of the control response (feces and isoflavone without any added bacterial strains). The reason for this normalization is that control degradation rates were different from one subject to another and from one isoflavone to another (Figure 8). Differences in control degradation rates obtained in the first and second set of experiments (higher mean glycitein degradation rate in nutrient poor for the first set of incubation and higher mean genistein degradation rate in nutrient rich for the second set of incubation) may be due to the fact that different fecal background were chosen for each set of incubation. Thus, making comparison among baseline degradation rates was impossible and the different patterns observed harder to interpret with certitude.

Results observed from the first set of incubations in nutrient poor media gave marginal or significant increases in degradation rates of all three isoflavones for almost every treatment (Figure 9). This fits with our hypothesis that a nutrient-poor media mimicking long

GTT resulted in increased isoflavone degradation compared to control when putative isoflavone-degrading microorganisms were added to the fecal suspension. However, we obtained for most of the data very large standard deviations. This was due to a difference in percentage of the control response from one individual to another for the same strain studied. Therefore, it seemed that, bacterial metabolic activities may be influenced by the fecal background matrix. Large interindividual differences in fecal isoflavone degradation rates expressed as % control response may fit with the known great variability among individuals in isoflavone bioavailability. Indeed, this process is not homogenous and showed great variation in urinary (up to 100-fold difference), plasma and fecal kinetics from one individual to another (Hendrich et al. 1998; Xu et al. 1994), hence these variations could then be due not only to certain bacterial species present in the gastro-intestinal tract, but also to the level of metabolic and enzymatic activities of these microbial species influenced by the gastro-intestinal environment between individuals. The fact that bacteria in combination produced the same range of increase in isoflavone degradation rate compared to single strains showed that there may not be an additive effect when different were added in the same treatment tube. It seemed that the putative isoflavone degrading strains may compete for isoflavones, resulted in no increase in degradation rate by multiple strains compared to a single strain added to the fecal mixture.

The first set of incubations in nutrient-rich media gave a much smaller percentage increase in isoflavone degradation rate expressed as a percentage of the control response compared to that in nutrient poor media (Figure 10). These results fitted with our hypothesis that a nutrient rich media representing a rapid gut transit time resulted in lower response of isoflavone degradation by putative isoflavone-degrading microorganisms when compared to nutrient poor conditions. The highest mean increase was 67% of control response in genistein degradation for *P. ruminicola* in rich media. We observed inhibitory effects of a nutrient rich media on glycitein degradation rates (the presence of *B. eggerthii* showed a significant decrease compared with control response). Trends towards statistical significance ( $0.1 < p < 0.05$ ) were also observed with some daidzein degradation rates (*Bacteroides*, *Bacteroidales*, *P. pallens* and *B. eggerthii*) and one genistein degradation rate (*Bacteroides*). These trends observed will deserve further investigation with greater sample size (Figure 10).

Due to a more homogenous effect that led to a smaller standard deviation in nutrient rich media, significant increased daidzein degradation rates were observed with the presence of *Prevotella* (mean increase 23%) as well as genistein with *P. pallens* (mean increase 33%) when compared to the control response in nutrient rich media. *P. pallens* produced a significant increase in genistein degradation when in a nutrient-rich media led us to hypothesize that some strains are capable of influencing isoflavone degradation even though nutrient availability is rich, partly explaining why individuals with rapid gut transit time (higher colonic nutrient availability) do degrade isoflavone to some extent.

Control degradation rates in the second set of incubations gave different results from the first set of incubations (Figure 8). Genistein degradation was significantly higher in nutrient rich media while glycitein degradation was higher in nutrient poor during the first set of experiment. Considering that different fecal matrix were considered for each set, statistical comparison of baseline degradation rates between both sets were impossible to make. However, the fact that the same subject investigated in both sets two months apart (Caucasian male) had degradation rates that followed the overall results obtained with an n of 3 for each set of incubations supported our preliminary observation that degradation rates studied even one month apart may show great variation, probably because of differences in microbial profiles and isoflavone-degrading microorganisms already present in the control fecal samples. Therefore, the fecal matrix and bacterial ecology may be of importance in determining bacterial adaptation and degradation of isoflavones. Even though gut bacterial ecology is thought to be stable over time (Fuller and Perdígón. 2003) our result on DGGE in the same individual one month apart (Figure 2) showed some differences in band profiles so that colonic factors may influence not only bacterial metabolism, but also bacterial profile, even over short periods of time, which ultimately would influence isoflavone disappearance. This phenomenon was also observed by Zheng et al. (2003) who investigated stability of isoflavone degradation phenotypes in Asian (n=35) and Caucasian (n=31) at two time points five months apart. 22/35 Asian and 17/31 Caucasian switched their daidzein disappearance phenotypes, while 11/35 Asian and 16/31 Caucasian switched their genistein disappearance phenotypes. Low degraders were more stable between both time points whereas moderate and high degraders were less stable. Indeed, while 4 Caucasians were identified as high

degraders at time 0, none were identified in this cluster 5 months later. In the present study, we selected moderate or moderate borderline low daidzein degraders so that these individuals may have been more susceptible to modify their degradation phenotype, which may provide some understanding in the differences observed in control rates in the first and second set of incubations (figure 8). In addition, the subject selected for the intra-individual variation study was previously assessed as overall moderate degrader for all three isoflavones. Variation in glycitein disappearance may be due to a baseline phenotype apparently less stable over time (Zheng et al. 2003). Finally, this would also agree with the data obtained from the same individual (male Caucasian) selected for both sets of incubations in nutrient poor and rich media. His baseline rates followed those obtained with an n of 3, where variations in glycitein and genistein degradation were noticed.

Results obtained from nutrient-poor incubations in the second set of incubations showed similar results to those obtained in the first set of treatments (Figure 9). This confirmed our hypothesis that a nutrient-poor media mimicking long GTT resulted in increased isoflavone degradation compare to control when putative isoflavone-degrading microorganisms were added to the fecal suspension. In addition, the range of increased percentage control response varied tremendously, with 3 strains influencing to greater extent isoflavone degradation rates: *B. acidifaciens*, *B. uniformis* and *T. forsythensis*. Isoflavone degradation rates obtained with the presence of *P. veroralis* and *C. orbiscidens* showed intermediate increase between those produced with the three strains influencing degradation the greatest and other strains that produced a lower mean percentage increase compared to control. The fact that we observed a greater range of increase degradation as percentage control response with these bacteria and compared to the strains investigated during the first set of incubations led us to hypothesize that some strains may actually be more efficient in influencing isoflavone degradation than others.

In nutrient rich conditions, *B. ovatus*, *B. acidifaciens*, *E. ramulus*, *C. orbiscindens* and *T. forsythensis* showed significantly increased isoflavone degradation rates (up to 600% of control response for *B. acidifaciens*) which were different from the first set of incubation in which only *P. pallens* was a significant genistein degrader (Figure 10). Overall, we were not able to identify a specific relationship between presence of a putative strain and degradation



of a specific isoflavone. It seemed that these species were capable of degrading at least two isoflavones. Only *P. pallens* seemed to be more specific to genistein degradation ( $p < 0.05$  when compare to control response in the first set of incubation) despite a trend in increasing glycitein and daidzein degradation rates as well ( $0.05 < p < 0.08$ ). *B. uniformis* was not investigated in nutrient rich media, but considering the high increase in isoflavone degradation rate in nutrient-poor media similar to *B. acidifaciens* and *T. forsythensis*, we expect that the presence of this species would cause significant isoflavone degradation in nutrient rich media. This deserves further investigation. Identifying *E. ramulus* as one of the key species in influencing isoflavone degradation is in agreement with Schoeffer et al. (2002). But in our hands, *E. ramulus* did not degrade isoflavones as a single strain. When using different media and in a fecal matrix, *E. ramulus* increased significantly isoflavones. *C. orbiscindens*, shown by Schoeffer et al. (2003) to degrade flavonoids was also one of the main species that we identified as having a significant effect in degrading isoflavone. These results did not confirm our hypothesis that a nutrient rich media representing a rapid gut transit time resulted in lower response of isoflavone degradation by putative isoflavone-degrading microorganisms when compared to nutrient poor conditions. However, it confirmed what was found with *P. pallens* in the first set of incubations. Some species may influence isoflavone degradation, regardless of the nutrient availability. This would suggest that if these species do actually degrade isoflavones, lower population of these strains in low degraders and higher population in high degraders would agree with the results found with PCR-DGGE (figures 2, 5 and 6). Species increasing significantly isoflavones degradation in nutrient poor only may suggest that a wider range of bacterial species would degrade isoflavones under these circumstances, explaining partly why individuals with long gut transit time degrade isoflavone to a greater extent (more species involved). A narrower range of species were found to increase isoflavone degradation in nutrient rich, which in turn may explain the fact that individuals with rapid gut transit time have lower isoflavone degradation rates (less species involved)

An interesting finding constant throughout this project was that variations in percentage degradation rate over control response with feces and isoflavones only did not correlate with baseline control rates, whether it was in nutrient rich or poor. Therefore, the

data obtained with fecal incubation and species selected were independent of baseline isoflavone degradation rates.

From observations that some bacterial species were capable of increasing significantly isoflavone degradation whether the media was rich or poor in nutrients, we suggested that these strains may be responsible for isoflavone degradation in high and low degraders with the assumption that high degraders have greater amounts of these species compared to low degraders, as reflected in DGGE analysis. High degradation pattern associated with lower nutrient availability may also be explained by a greater number of species degrading isoflavones that might be used as an alternate source of nutrient for these species to survive and compete. However, the actual relationship of these species using isoflavone as a nutrient source deserves more investigation. Our data suggest that the presence of these species increased isoflavone degradation, but did not prove with certitude that these same species were actually capable of using isoflavone as a nutrient source.

Last, but not least, one of the most difficult factors to include in such a design would be bacterial competition over time. Some fecal bacterial species may be more prone to compete with the added bacteria, resulting in a lower (or possibly higher) isoflavone degradation rate but this remains to be further investigated. Thus, bioavailability of isoflavone must include a complex system of parameters that must be identified and controlled and in which each individual may have to be considered separately in order to fully understand how microbial metabolism and composition influence isoflavone degradation.

## **SUMMARY AND RECOMMENDATIONS**

As a summary, our *in vitro* incubations provided us with strong basis to study isoflavone disappearance by putative isoflavone-degrading microorganisms. The presence of a fecal background was of greater relevance to an *in vivo* situation compare to single strain incubations, where no bacterial competition occurs. Selecting moderate degraders in the present study for nutrient rich and poor experiments needs to be reconsidered for future experiments. Too much variability in baseline degradation rates indicated that selecting low

degraders may be more appropriate, because these individuals may have a more stable flora over time (Zheng et al. 2003). Reproducibility of incubation treatments with added bacteria may also be greater with fecal backgrounds from low degraders. On the other hand, moderate degraders may be of greater interest to study short-term changes in gut microflora and how these changes would affect isoflavone degradation. Significant data obtained here with one individual would deserve further attention with greater number of moderate degraders. It would be also of interest to study high degraders and how the species identified in the present study would affect isoflavone degradation. If high degraders have significantly greater amounts of strains identified in the present study, we may expect a lower response from adding the same microorganisms. This would also lead to study dose-response type of experiment to assess the minimal bacterial concentration needed to significantly modify the response in degradation compare to control. Screening bacterial species from other families could be of importance as well. If no significant variations in degradation occur with other strains, it would limit our focus to the Bacteroidales (*Bacteroides* & *Prevotella*) and Clostridiales groups. Finally, finding a bacterial strain that would not alter significantly the original fecal microflora, nor control degradation rates would lead to the establishment of a negative control to better assess data from treatments.

## CONCLUSION

The present study confirmed the significant effect of several bacterial species identified through PCR-DGGE on isoflavone degradation in human high and low fecal degraders. Many questions about gut microbial isoflavone metabolism remain unsolved. The complexity of the fecal microflora, coupled with many aspect of gut microbial ecology that have not been elucidated make our conclusions preliminary.

Aside from *B. uniformis* that will deserve further attention, we concluded that in this study, the main species that influenced isoflavone degradation were *B. ovatus*, *B. acidifaciens*, *E. ramulus*, *C. orbiscindens*, *T. forsythensis* and possibly *P. pallens*. This conclusion is based on the fact that isoflavones were significantly more degraded in the presence of these species regardless of the nutrient environment, which would explain not

only why all individuals degrade isoflavones but also the higher presence of these species in high degraders compared to low degraders as reflected on DGGE analysis. Other species involved in a high degradation in vitro simulation only may also indicate that a greater number of species may explain high isoflavone degradation. Considering that subjects selected for this study were from different backgrounds, it would be helpful to re-investigate isoflavone degradation in nutrient-rich and poor media with fecal background of individuals from carefully defined population subgroups. Thus, factors that must be considered in future studies to fully understand microbial metabolism of isoflavone include: sex, ethnicity, age, gut transit time, stability of fecal isoflavone degradation phenotype and dietary habits. Further studies should also include dose-response analysis of the different strains identified here with respect to isoflavone degradation.

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Figure 1: overview of PCR-DGGE protocol and methods

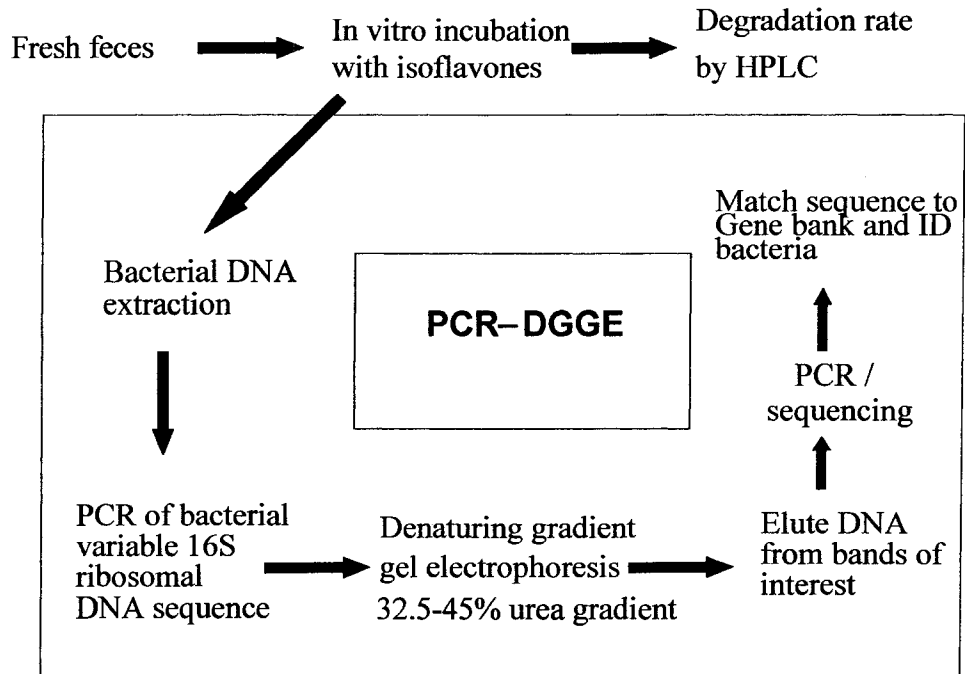




Figure 2: DGGE (32.5-45% urea gradient) of fecal DNA from the same individual over two months and associated glycitein degradation rates. Microbial profiles are shown in duplicates for each time point. Bands of interest are noted as A through G.

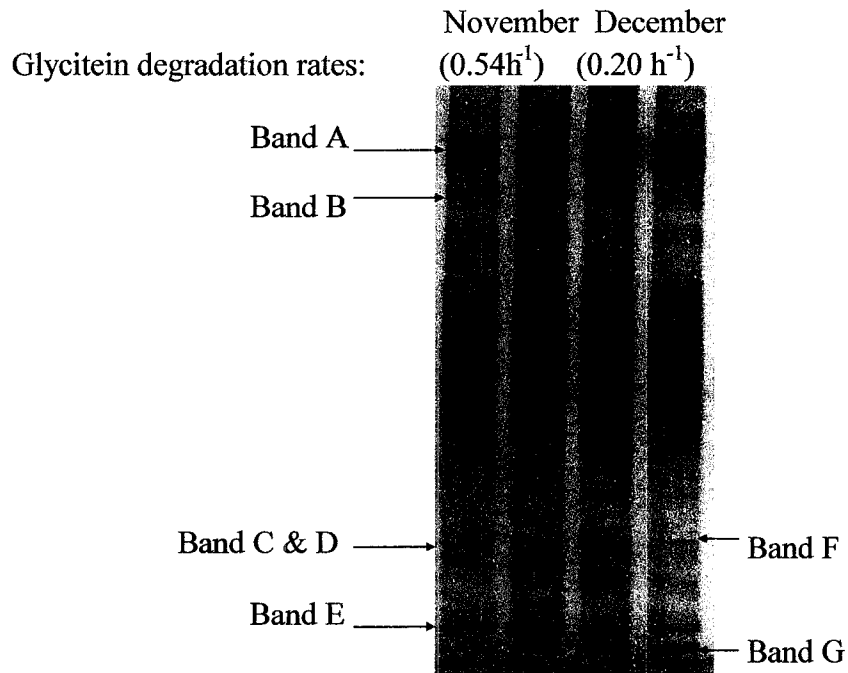


Figure 3: Segmented (0-6h and 0-12h) genistein degradation rates from in vitro fecal degradation screening of subjects showing 3 patterns of degradation (stable, decrease and increase).

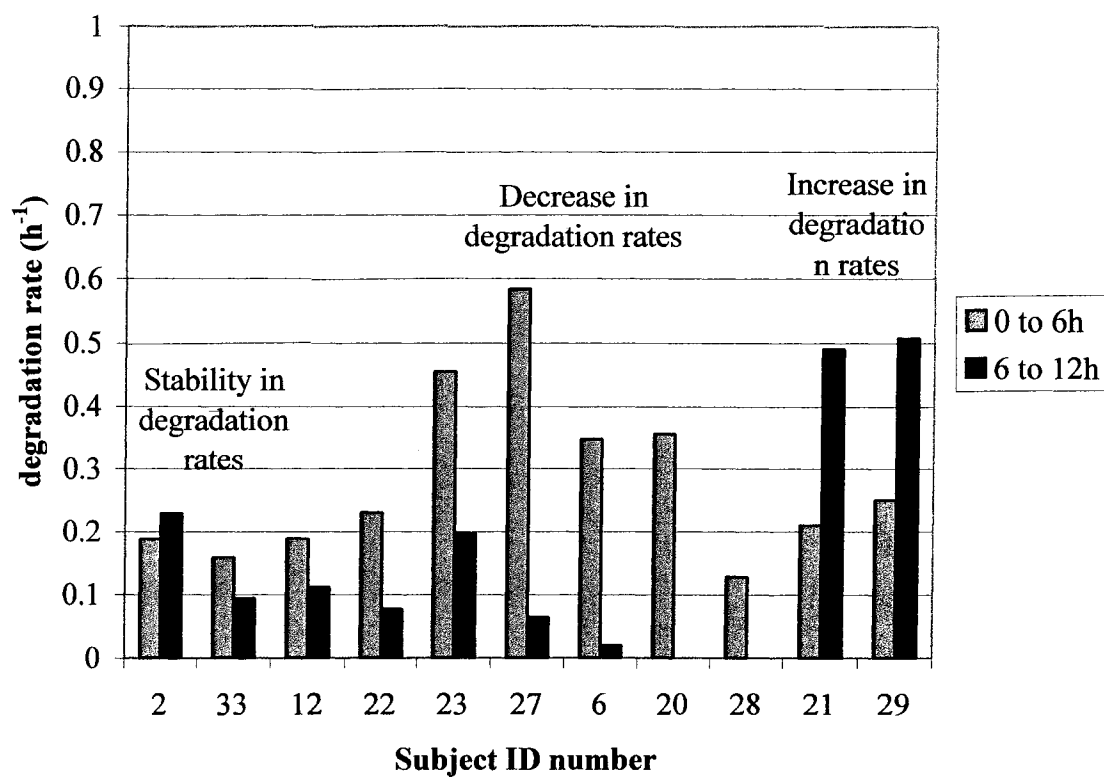


Figure 4: DGGE profile (35-50% urea gradient) of fecal microbial 16S rRNA variable region DNA sequences from the same individual at 0 and 12h of in vitro incubation in treatment (feces + isoflavone) and control (feces only).

Treatment Control  
0h 12h 0h 12h



Figure 5: DGGE profile (32.5-45% urea gradient) of fecal microbial 16S rRNA variable region DNA sequences from low (first 4 lanes) and high (last 4 lanes) glycine degraders and bands of interest (Gly 1 through Gly 5).

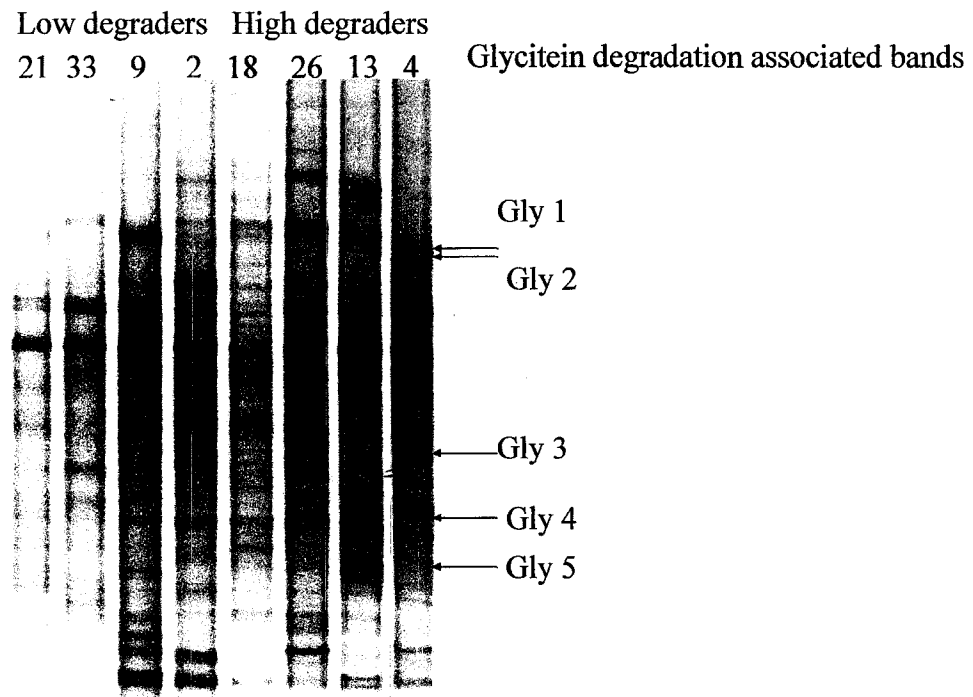


Figure 6: DGGE profile (32.5-45% urea gradient) of fecal microbial 16S rRNA variable region DNA sequences from low (first 4 lanes) and high (last 4 lanes) genistein degraders and bands of interest (Gen 1 through Gen 5).

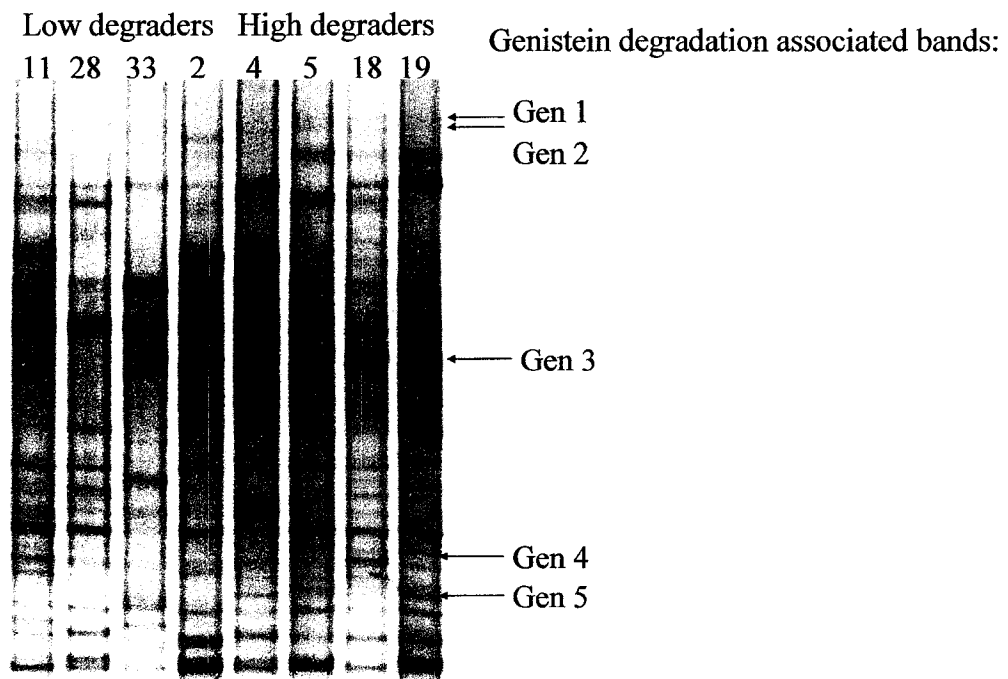


Table 1: Analysis of bands found in figure 2, 5 and 6: band intensity, overall taxonomy reports and bacterial identification

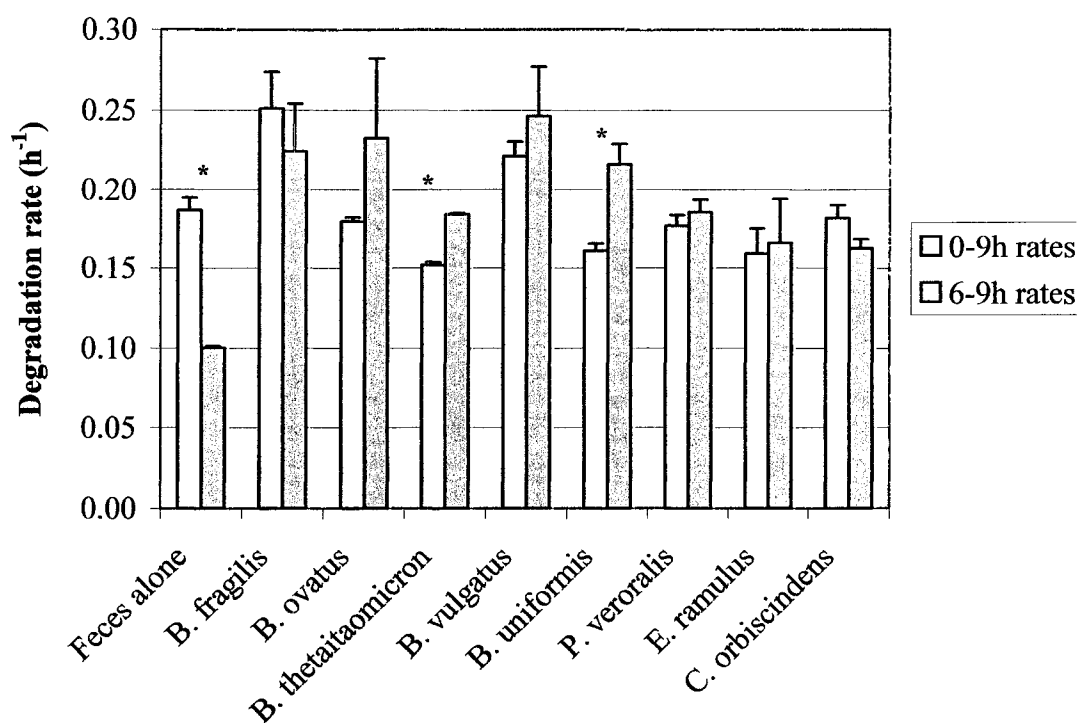
Band Identifier	Mean Low	Mean High	Taxonomy report	Bacterial
A <sup>b</sup>	104.5 ± 2.1	158.5 ± 4.8	63% unclassified 37% Bacteroidales	<i>Bacteroides fragilis</i>
B <sup>b</sup>	104.5 ± 2.1	131.5 ± 2.1	46% unclassified 54% Bacteroidales	<i>Porphyromonas gingivalis</i>
C <sup>b</sup>	95.0 ± 3.2	128.0 ± 1.4	73% unclassified 27% Bacteroidales	<i>Bacteroides vulgatus</i>
D <sup>b</sup>	94.0 ± 3.8	127.5 ± 1.3	65% unclassified 35% Bacteroidales	<i>Prevotella ruminicola</i>
E <sup>b</sup>	123.0 ± 2.8	170.0 ± 1.4	64% unclassified 36% Bacteroidales	<i>Prevotella pallens</i>
F <sup>b</sup>	118.0 ± 2.8	107.0 ± 2.8	50% unclassified 50% Bacteroidales	<i>Prevotella oralis</i> <i>Prevotella veroralis</i>
G <sup>b</sup>	142.5 ± 2.9	121.0 ± 2.8	50% unclassified 50% Bacteroidales	<i>Bacteroides ovatus</i>
Gen 1 <sup>c</sup>	107.5 ± 14.4	154.8 ± 13.8	42% unclassified 58% proteobacteria	
Gen 2 <sup>c</sup>	113.8 ± 14.9	159.5 ± 11.4	100% unknown	
Gen 3 <sup>c</sup>	171.3 ± 21.0	210.0 ± 10.8	56% unclassified 44% Bacteroidales	<i>Bacteroides uniformis</i> <i>B. thetaiotaomicron</i>
Gen 4 <sup>c</sup>	128.3 ± 14.0	168.3 ± 4.5	89% unclassified 6% Clostridiales 5% Fusobacterium	
Gen 5 <sup>c</sup>	120.0 ± 12.2	164.3 ± 14.9	75% unclassified 25% Clostridiales	<i>Fusobacterium prausnitzii</i>
Gly 1 <sup>c</sup>	120.5 ± 18.1	187.5 ± 22.5	57% unclassified 43% Bacteroidales	<i>Tannerella</i>
Gly 2 <sup>c</sup>	132.3 ± 36.7	177.5 ± 20.6	61% uncultured 39% bacteroidales	<i>Bacteroides eggerthii</i>
Gly 3 <sup>c</sup>	146.3 ± 16.0	180.3 ± 5.0	62% unclassified 38% Bacteroidales	<i>Bacteroides acidifaciens</i>
Gly 4 <sup>c</sup>	142.5 ± 17.6	183.8 ± 16.5	87% unclassified 13% Clostridiales	<i>Ruminococcus obeum</i>
Gly 5 <sup>c</sup>	141.5 ± 16.0	180.3 ± 5.0	100% unclassified	

a: All means within the same row are significantly different from each other at  $p < 0.05$ .

b: mean band intensity (by densitometry) ± standard deviation calculated from duplicate values for each time point.

c: mean band intensity (by densitometry) ± standard deviation calculated from the four individuals selected within each phenotype.

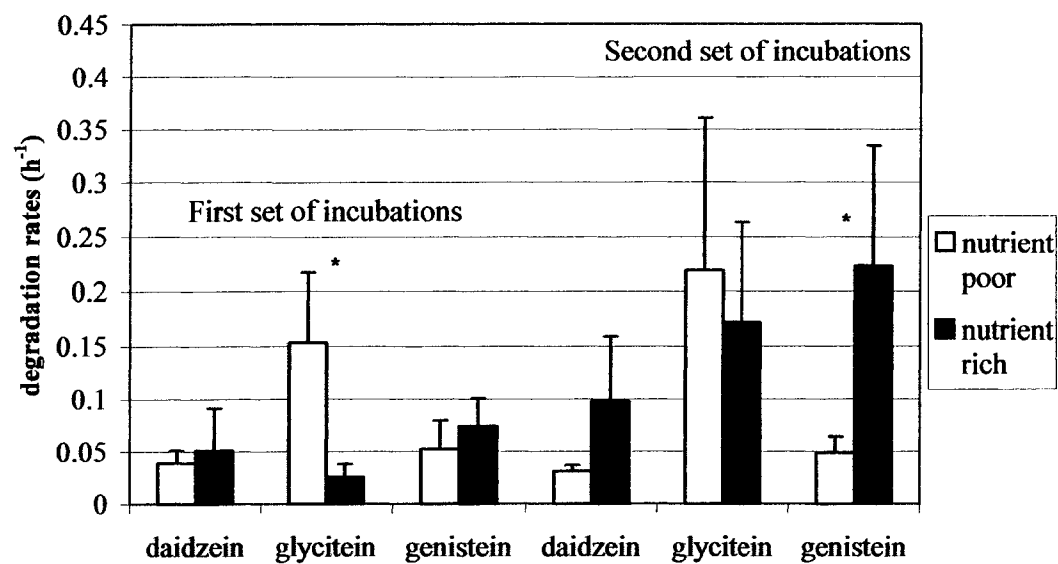
Figure 7: Genistein in vitro fecal degradation rates obtained between 0-9h and 6-9h incubation in control showing a significant decrease and treatments with selected species showing either stability or significant increase (n=1).



(\*  $p < 0.05$  between 0-9h and 6-9h rates).

Error bars obtained represent standard deviation from duplicate values within each treatment.

Figure 8: Mean control in vitro fecal degradation rates showing a significant decrease for glycitein (first set of incubations, n=3) and increase for genistein (second set of incubations, n=3) under nutrient rich conditions.



(\*  $p < 0.05$  between nutrient rich and nutrient poor mean rates  $\pm$  standard deviation).



Figure 9: Mean isoflavone in vitro fecal degradation rates expressed as percentage of control response (feces and isoflavone only) per  $10^9$  bacteria/mL media in nutrient poor media (Top: first set of incubations, n=3) (Bottom: second set of incubations; n=3). (\*p<0.05 when compare to control: feces and isoflavone only normalized at 100%).

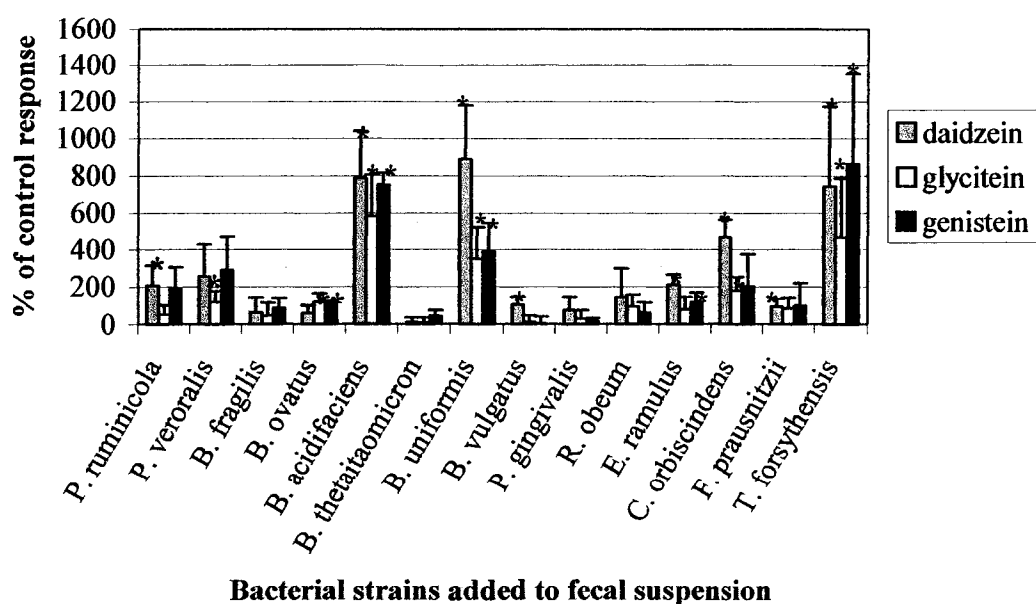
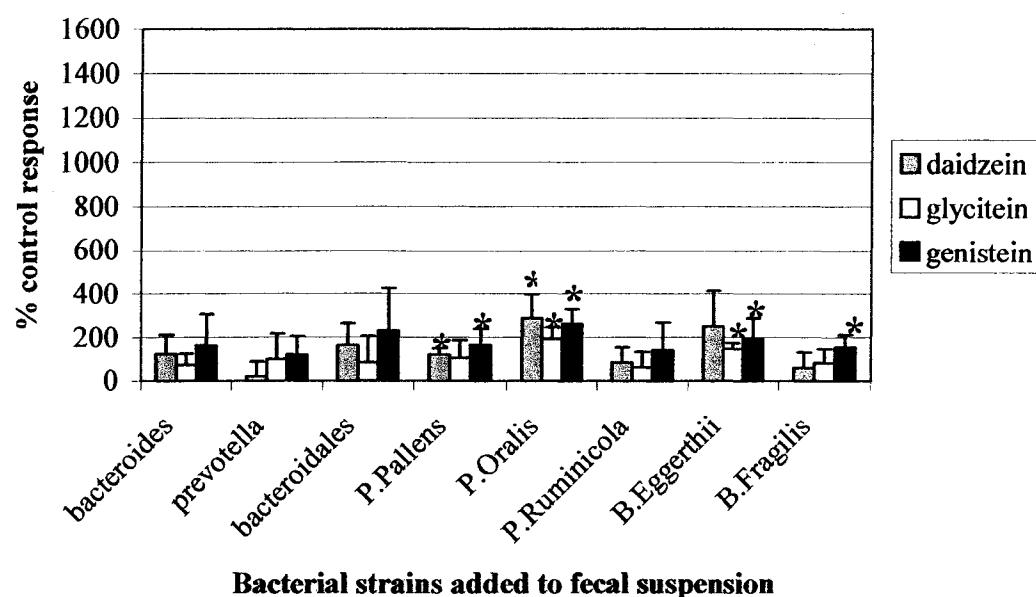
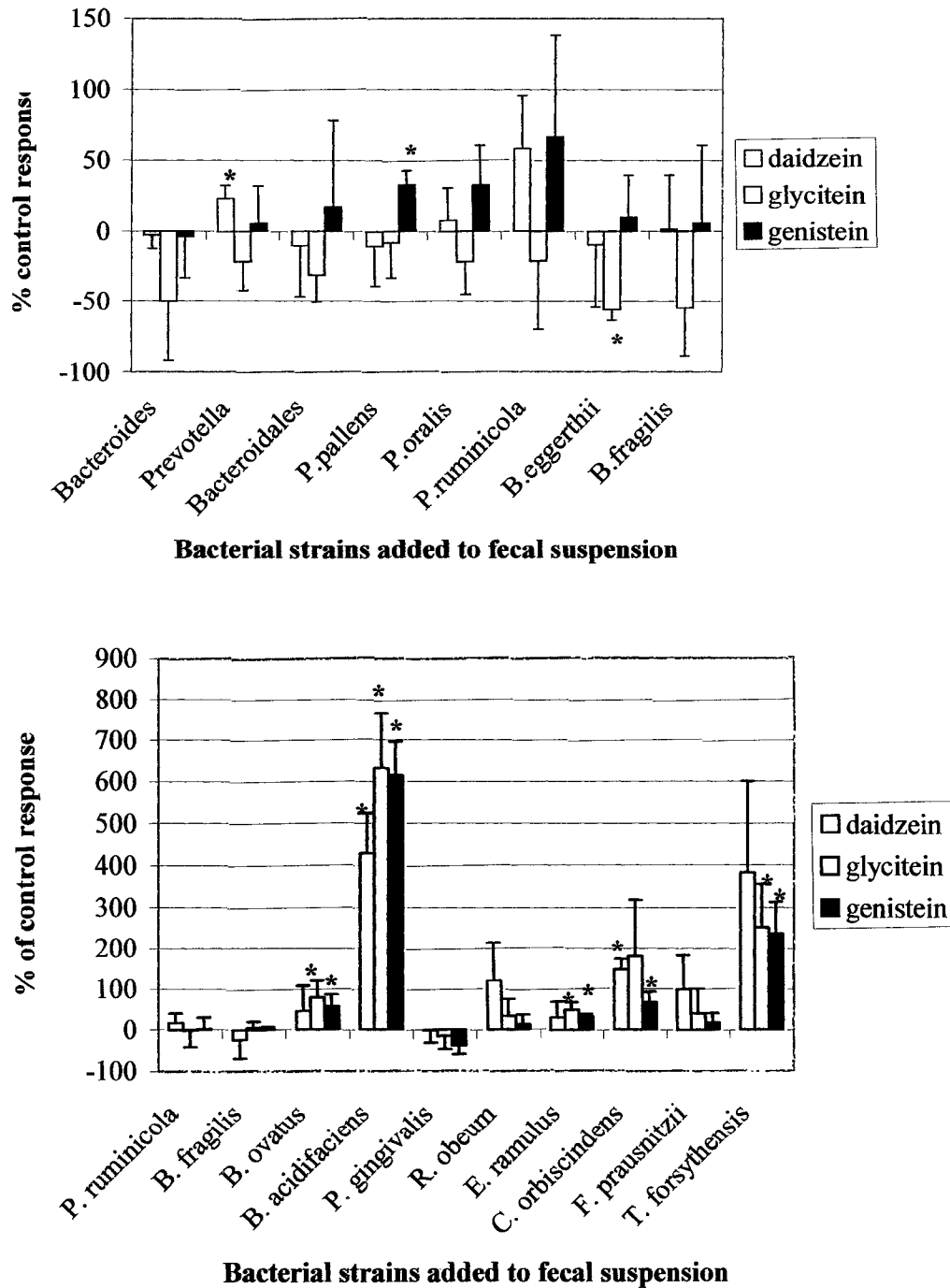


Figure 10: Mean isoflavone in vitro fecal degradation rates expressed as percentage of control response (feces and isoflavone only) per  $10^9$  bacteria/mL media in nutrient rich media (Top: first set of incubations, n=3) (Bottom: second set of incubations; n=3). (\*p<0.05 when compare to control: feces and isoflavone only normalized at 100%).



## GENERAL CONCLUSIONS

To better assess health-promoting effects of isoflavones and their prevention of chronic diseases, a more accurate understanding of isoflavone bioavailability must be also considered, in humans and animals. The studies performed here clearly showed how intricate and complicated the process of bioavailability is. In addition to the difficulties in drawing definite conclusions in humans, the establishment of a valid animal model close to humans is even more difficult, considering all the knowledge gaps that must be filled to fully appreciate the potential of these compounds.

When establishing bioavailability of isoflavones in Golden Syrian hamsters, we found several aspects similar to humans. Male hamsters are lower urinary isoflavone excretors than females. The range of urinary excretion in female hamsters is close to that of women whereas male hamsters have lower bioavailability than men. Daidzein was shown to be more bioavailable than genistein (similar to that in humans), while glycitein was somewhat intermediate (whereas glycitein is similar to daidzein in bioavailability in humans). Human *in vitro* fecal degradation of isoflavones is a good predictor of bioavailability. We found that in hamsters, a closer relationship was observed to apparent absorption with cecal rather than fecal *in vitro* isoflavone degradation rate. Finally, fecal isoflavone excretion is extremely low and does not correlate with urinary excretion. Therefore, considering similar ranges of urinary excretion sex and isoflavone difference and the relationship between *in vivo* and *in vitro* cecal data, hamsters may be a good model of isoflavone bioavailability that might be useful in simulating long term effects of human variability in gut microbial degradation of isoflavones on isoflavone health effects.

Another major aspect investigated in the present project was to identify isoflavone-degrading microorganisms, a major aspect of the overall pattern of isoflavone bioavailability. Using molecular and microbiological techniques as well as new *in vitro* simulations that were hypothesized to mimic *in vivo* gut microbial conditions associated with rapid and long gut transit time, we found that *B. ovatus*, *B. acidifaciens*, *E. ramulus*, *C. orbiscindens*, *T. forsythensis* and possibly *P. pallens* and *B. uniformis* used isoflavones as a primary source of nutrients, thus playing a major role in degrading these compounds as a ubiquitous action,

possibly explaining why isoflavone degradation occurs in every individual. However, different proportions of these strains among individuals may explain the wide range of isoflavone degradation, leading people to be phenotyped as high, moderate and low degraders. In addition, a wide range of other strains degraded isoflavones especially in nutrient poor media, suggesting that a poor colonic nutrient availability may permit gut microbes to degrade isoflavones, species that may differ from one individual to another, considering that some microbial species were able to greatly increase isoflavone degradation in one subject whereas different microbial species may be involved in additional degradation in another individual.

As a conclusion, isoflavone bioavailability is a complex process and it will take many more years to fill all the knowledge gaps of this particular research area. It will be extremely challenging to find an animal model showing bioavailability patterns that would exactly mimic what is known in humans. Hamsters, because of close cholesterol metabolism and also great similarities in bioavailability compared to humans may be one of the most appropriate animal models to study isoflavone health-related effects. Difficulties in finding a valid animal model are also related to the fact that isoflavone bioavailability is not fully understood in humans. Identifying isoflavone-degrading human gut microorganisms would fill one of the main knowledge gaps in this area. In this project, we identified with great confidence 5 strains and possibly 2 more that are involved in degrading isoflavones in the human gut. Greater proportion of these strains in high degraders coupled with other secondary strains that may be involved in degradation when nutrient availability is low is relevant to the large range of degradation rates observed among humans.

## ACKNOWLEDGMENTS

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